THE SPINAL RELEASE OF IMMUNOREACTIVE NEUROPEPTIDE Y IN RATS WITH A PERIPHERAL NERVE INJURY

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DECLARATION

I hereby declare that the composition of this Thesis and the work presented in it are entirely my own with the exception of setting up the neuropathic model and the behavioural studies which were carried out in collaboration with Dr L.A. Colvin. This thesis has not been submitted for the purposes of obtaining any degree or qualification from any other academic institution.

Margo Anne Mark

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SUMMARY

The functional significance of neuropeptide Y in the rat spinal cord is unclear and there have been no reports of the stimuli needed to produce release of this peptide *in vivo*. When a peripheral nerve is injured a *de novo* synthesis of neuropeptide Y occurs in many large and some medium sized dorsal root ganglion neurones. The experiments in this thesis employed the antibody microprobe technique to study both in normal rats and in those with a peripheral mononeuropathy the spinal release of extracellular immunoreactive neuropeptide Y and to determine the origin of such release.

In the initial experiments, microprobes bearing immobilised antibodies to neuropeptide Y were inserted into the lumbar spinal cord of urethane anaesthetised normal rats. In the absence of peripheral stimuli microprobes detected a high basal presence of immunoreactive NPY throughout the entire dorsal and ventral horn. Electrical stimulation of large diameter afferents of the ipsilateral sciatic nerve and unmyelinated primary afferents did not significantly alter the spinal release of immunoreactive neuropeptide Y in the spinal cord. Transection of the spinal cord at a low thoracic level resulted in increased levels of immunoreactive neuropeptide Y only in the lower ventral horn. The predominant failure of electrical stimulation and of spinalisation to significantly alter the basal levels of immunoreactive neuropeptide Y suggests that the latter results from spontaneous activity in intrinsic neurones.

For studies of rats with a peripheral mononeuropathy, the model of Bennett & Xie was used. Postoperatively the development of mechanical allodynia and hyperalgesia were assessed and animals used at 10-14 days only if they displayed the characteristic behavioural signs associated with this model. Sham experiments were carried out in separate animals as controls for the effect of surgery per se. In sham animals both sides of the lumbar spinal cord showed a significant spinal release of immunoreactive neuropeptide Y throughout the entire dorsal horn. The site of

greatest extracellular levels was the superficial dorsal horn. A similar distribution was also found in the neuropathic animal on the side contralateral to the nerve ligation. On the ipsilateral side of the neuropathic rat however there was a further zone of spontaneous release of immunoreactive neuropeptide Y in the mid and lower dorsal horn (approximating to laminae III, IV and V). Additionally, electrical stimulation of large diameter afferents of the ipsilateral sciatic nerve resulted in an increase in the spinal release over the entire dorsal and upper ventral horn. Stimulating both large diameter myelinated afferents and small unmyelinated afferents did not increase release of immunoreactive neuropeptide Y above that observed by stimulating large myelinated fibres alone. Thus, the additional zone of spontaneous release found on the ipsilateral side of the neuropathic rat probably represents spontaneous activity in damaged and/or regenerating primary afferents. This may represent central release of a neuroactive compound by ectopic impulses in such fibres.

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ABBREVIATIONS

aPP	avian pancreatic polypeptide
ATP	adenosine triphosphate
Ba^{2+}	barium
BIBP3226	R-N^2-(diphenylacetyl)-N-(4-hydroxyphenyl)methyl-arginamide
bPP	bovine pancreatic polypeptide
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic AMP
CCD	charged coupled device
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CPP	3-(2-carboxypiperazin-4-yl)propyl-1 phosphonic acid
CRPS	complex regional pain syndromes
cDNA	complementary deoxyribonucleic acid
Ci	Curies
CNS	central nervous system
COMT	catechol -o-methyltransferase
cpm	counts per minute
[^{3}H]DTG	1,3,-di-o-tolyguanidine
EDRF	endothelium-derived relaxing factor
GAP-43	growth associated protein-43
GABA	gamma amino butyric acid
GTP	guanine triphosphate
G-protein	guanyl nucleotide binding protein
HE90481	N^1-[3-(3,5,-difluorophenyl)-3-(pyridin-2-yl)propyl]-N^3-[3-1H-imidazol-4-yl)propyl-guanidine trihydrochloride

HEL	human erythroleukaemia cells
β-HRP	Cholera toxin (B-fragments) conjugated to horseradish peroxidase
5-HT	5-hydroxytryptamine
IASP	International Association for the Study of Pain
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IP_3	inositol triphosphate
i.r.	immunoreactive
i.t.	intrathecal
i.v.	intravenous
K^+	potassium
LDCV	large dense core vesicles
mg ; µg	milligram ; microgram
ml ; µl	millilitre ; microlitre
mm ; µm	millimetre ; micrometre
mRNA	messenger ribonucleic acid
MK-801	dizocilpine maleate
NA	noradrenaline
Na^+	sodium
NMDA	*N*-methyl-D-aspartate
NPY	neuropeptide Y
PACAP	pituitary adenylate cylase activating polypeptide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2 ; PGI_2	prostaglandins
PNS	peripheral nervous system
PPT	preprotachykinin
PTX	pertussis toxin

PVN	paraventricular nucleus
PYX1	Ac-[3-(2,6-dichlorobenzyl)Tyr27,D-Thr32]NPY-(27-36)amide
PYX2	Ac-[3-(2,6-dichlorobenzyl)Tyr27,36, D-Thr32]NPY-(27-36)
PYY	peptide YY
RIA	radioimmunoassay
RSD	reflex sympathetic dystrophy
SC19920	1-acetyl-2-(8-chloro-10,11-dihydrodibenz [b.f.] oxazepine-10-carbonyl) hydyazine
SEM	standard error of the mean
SIP	sympathetically independent pain
SKF-10047	N-allynormetazocine
SMP	sympathetically maintained pain
SP	substance P
SR120819A	(1[2-[2-[2-napthylsulfumoyl)-3-phenyl-propionamido]-3-[4-[N-(dimethylaminomethyl)-cis-cyclohexyl-methyl]amidino]phenyl]-pyrolidine (R.R.) steroisomer
SRR0107A	1-(2-[2-{2-napthylsulphamoyl}-3-phenyl-propionamido]-3-{4-{N-(4-[dimethylaminomethyl]-cis-cyclohexylmethyl)amidino}-phenylpropionyl)-pyrrolidine, (R,R) steroisomer
TEA	tetraethylammonium
1229U91-	([2',4],[2,4'] Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$ where Dpr = diaminopropionic acid
VIP	vasoactive intestinal polypeptide
VMN	ventromedial nucleus

CHAPTER 1: Introduction to Neuropeptide Y

1. NEUROPEPTIDE Y

The ultimate aim of this work is to contribute to the understanding of neuropathic pain which arises in the absence of an adequate stimulus and occurs in both man and animals. Animal models have been developed which may help our understanding of this clinical syndrome. However, before considering these models and the changes which follow peripherally and centrally, it is first necessary to describe the literature of the neuropeptide of interest in this study, neuropeptide Y.

1.1. SYNTHESIS AND STRUCTURE OF NEUROPEPTIDE Y

1.1.2. THE ORIGINS OF THE PANCREATIC FAMILY

In 1968 during the purification of insulin isolated from the chicken pancreas, avian pancreatic peptide (aPP, a 36 amino acid peptide) was discovered as a by-product (Kimmel et al, 1968; Kimmel et al, 1975). Avian PP was detected in pancreatic extracts of a number of birds and reptiles by radioimmunoassay (Langslow et al, 1973). Cells containing aPP were found to share many features with other known peptide hormone producing cells such as the presence of cytoplasmic granules and the ability to take up and decarboxylate administered amine precursors (Larsson et al, 1974). This peptide was found to be present in the pancreas of a number of avian and reptilian species by a later study (Larsson et al, 1976). Subsequently, a 36 amino acid was isolated from bovine pancreas and was found to be homologous to avian pancreatic peptide for 15 of the 36 amino acids (Lin & Chance, 1974). A peptide with structural similarities to avian pancreatic peptide was isolated from porcine duodenum by Tatemoto & Mutt (1978). They described a method for

detecting the presence of peptide amides using a chemical technique based on the fragmentation of peptides in tissue extracts and subsequent chromatographic identification of the resulting C-terminal alpha amide fragments. This sequence was called peptide YY (PYY) by Tatemoto & Mutt, (1980) because of its N and C-terminal tyrosines (Y being the abbreviation for tyrosine in the single amino acid code). Sequence similarities to pancreatic peptide are observed in the entire PYY molecule, 18 identical positions to porcine and bovine PP and 19 identical positions to avian PP. Tatemoto (1982a) therefore proposed that PYY and PP together formed a new peptide family. At first, PYY was also thought to be the PP-like peptide of the brain. The isolation from brain extracts of a peptide amide showed however that the peptide present in the brain was different from PYY. This peptide while having structural and biological similarities to both PYY and PP was previously uncharacterised. It was found to have a tyrosine at both N and C terminals of the molecule and was named neuropeptide Y (Tatemoto et al, 1982; Tatemoto, 1982b). In addition, to having identical N and C terminals as PYY, NPY has an equal number of Ala, Arg, Gly, His, Lys, Pro, Thr and Tyr residues (Tatemoto, 1982b). It was suggested that NPY was only present in nerve cells and PYY only in endocrine cells (Tatemoto et al, 1982). Early experiments investigating the coexistence of NPY with catecholamines in the central nervous system employed antibodies to avian PP (Hunt et al, 1981a; Lundberg et al, 1980), bovine pancreatic polypeptide (bPP) (Olschowka et al, 1981) and later to NPY (Hokfelt et al, 1983).

It became apparent that the endogenous peptide present in catecholamine neurones was neither aPP nor bPP but was more likely to be NPY (Everitt et al, 1984). DiMaggio and co-workers demonstrated that the PP like immunoreactivity previously observed in the brain was in fact due to the cross-reactivity of the antibody used with NPY (DiMaggio et al, 1985). Immunoreactive PYY was also found to be present in the CNS especially in the brainstem, various hypothalamic areas and in the autonomic ganglia (Bromme et al, 1985; Ekman et al, 1986; Happola et al, 1990).

Table 1. Amino acid sequence of members of the pancreatic polypeptide family

This shows the amino acid sequence of members of the pancreatic polypeptide family and has been adapted from Tatemoto (1982a & b). Amino acids have been highlighted in the sequences of avian (aPP), bovine (bPP), porcine (pPP) & porcine peptide YY (PYY) where similar to the sequence of porcine neuropeptide Y (NPY).

Amino acid sequence of members of the pancreatic polypeptide family

Peptide	Sequence
pNPY	Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-IleThr-Arg-Gly-Arg-Tyr-NH_2
bPP	Ala-**Pro**-Leu-Glu-**Pro**-Glu-Tyr-**Pro-Gly**-Asn-**Asp-Ala**-Thr-Pro-**Glu**-Gln-Met-**Ala**-Gln-**Tyr**-Ala-Ala-Glu-**Leu-Arg**-Arg-**Tyr-Ile-Asn**-Met-Leu-**Thr-Arg**-Pro-**Arg-Tyr**-NH_2
aPP	Gly-**Pro-Ser**-Gln-**Pro**-Thr-Tyr-**Pro-Gly**-Asp-**Asp-Ala-Pro**-Val-**Glu-Asp-Leu**-Ile-**Arg**-Phe-**Tyr**-Asp-Asn-Leu-Gln-Gln-**Tyr**-Leu-**Asn**-Val-Val-**Thr-Arg**-His-**Arg-Tyr**-NH_2
pPP	Ala-**Pro**-Leu-Glu-**Pro**-Val-Tyr-**Pro-Gly**-Asp-**Asp-Ala**-Thr-Pro-**Glu**-Gln-Met-**Ala**-Gln-**Tyr**-Ala-Ala-Glu-**Leu-Arg**-Arg-**Tyr-Ile-Asn**-Met-Leu-Thr-Arg-Pro-Arg-Tyr-NH_2
pPYY	**Tyr-Pro**-Ala-**Lys-Pro**-Glu-Ala-**Pro-Gly-Glu-Asp-Ala**-Ser-Pro-**Glu**-Glu-**Leu**-Ser-**Arg-Tyr-Tyr**-Ala-Ser-**Leu-Arg-His-Tyr**-Leu-**Asn-Leu**-Val-**Thr-Arg-Gly-Arg-Tyr**-NH_2

The distribution of ir-NPY has also been found to be present in the peripheral and central nervous system where it has many physiological roles (see section 1.2.).

1.1.3. THE TERTIARY STRUCTURE OF NEUROPEPTIDE Y

X-ray crystallography has been used to determine the tertiary structure of pancreatic polypeptide (PP; Blundell et al, 1981; Glover et al, 1983). The peptide has a globular structure, with NH_2 terminal residues 1-8 assuming a left handed polyproline II-like helix that is closely packed through hydrophobic interactions against an alpha-helix consisting of residues 13-32 to form a hydrophobic core. Studies of the structure of NPY are based on the sequence homology of this peptide with pancreatic peptide (PP). Comparison of the possible structure of NPY with that of avian pancreatic peptide has shown that structural elements in PP are maintained in porcine NPY and porcine PYY (Allen et al, 1987; MacKerell et al, 1989). This model suggests that NPY preserves a similar compact tertiary structure characterised by hydrophobic interactions between a COOH-terminal amphiphilic alpha-helix formed by residues 13-32 and an NH_2-terminal polyproline II-like helix that also has amphiphilic character and that these two structures are connected by a beta-turn (Allen et al, 1987; MacKerell, 1988; MacKerell et al, 1989; Minakata et al, 1989). The tyrosine residue in position 1 and amino acid residues at position 20-36 are located in close proximity to each other thus the structure has been described as a hair-pin.

Another model has been proposed on the basis of the data obtained using circular dichroism and nuclear magnetic resonance spectroscopy (Saudek & Pelton, 1990). This study proposed that the N and C terminals are not in close proximity as the C-terminal segment folds into an amphiphilic alpha-helix between 11-36.

Furthermore, the tyrosine at position 36 in association with residues 32 and 34 stabilised the folded structure and contributed to the formation of the alpha helix (Saudek & Pelton, 1990) whereas the tyrosine at position 1 did not interact with the rest of the structure. This study suggested that the 3 proline residues located in the N-terminal segment of the molecule may exist in either the cis or trans confirmation conferring no regular structure to this segment of the molecule.

The majority of structure activity models favour the hair-pin like model. It has been found that deletion of tyrosine at position 1 markedly decreased the affinity of NPY for its receptor sites (MacKerrell, 1988; Quirion et al, 1990) and has consequently been suggested to interact with NPY receptors. In addition, it has been suggested that the tyrosine at this position stabilises the hair-pin like structure of NPY (McLean et al, 1990). It has also been shown that modifications which are believed to increase interactions between the N- and C-terminal alpha helix and stabilise the hair-pin like structure, increase the affinity of these analogues for NPY receptor binding sites (Minakata & Iwashita, 1990).

1.1.4. OVERVIEW OF PEPTIDE SYNTHESIS AND METABOLISM

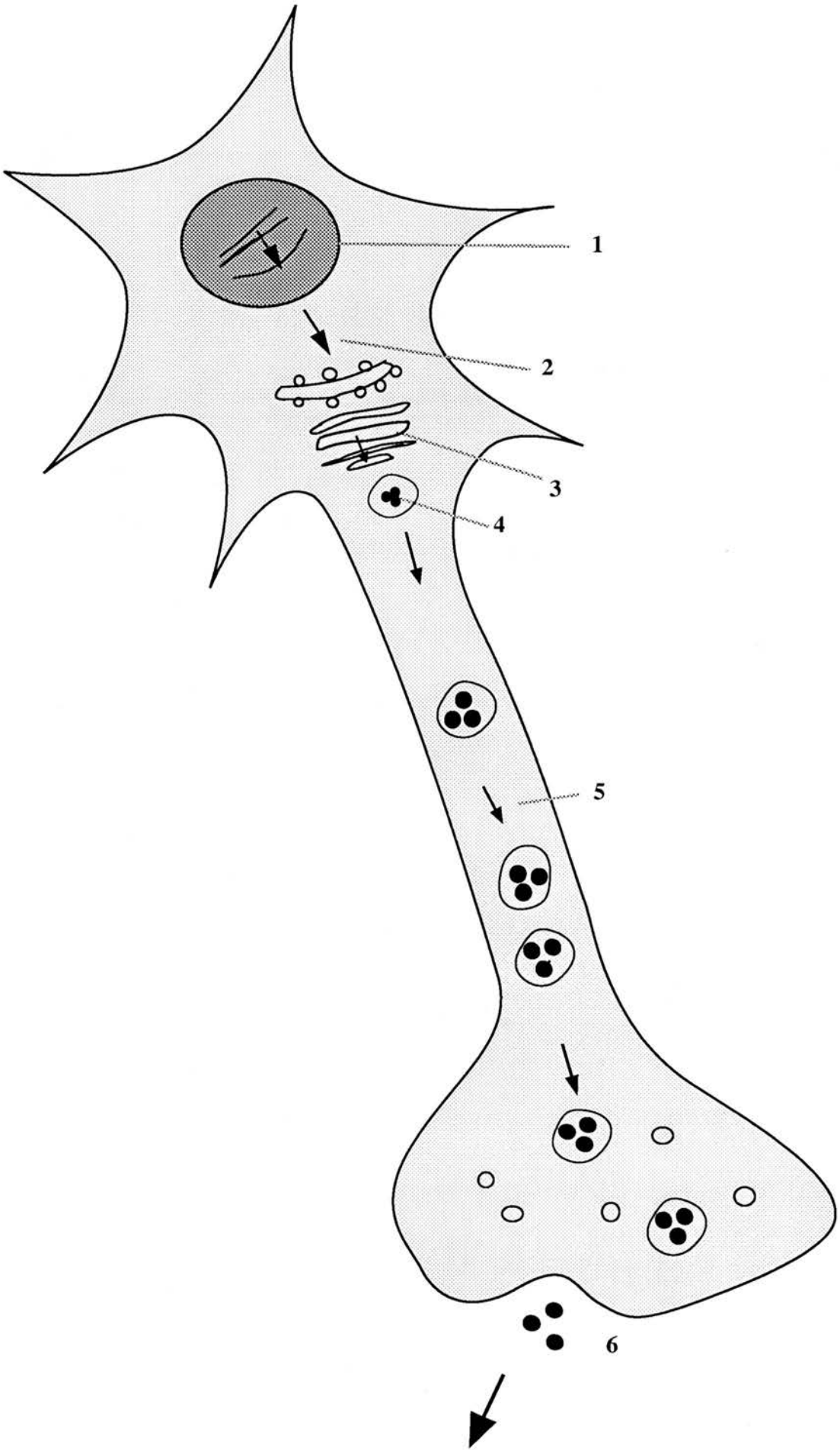
Unlike classical neurotransmitters which are synthesised in nerve terminals by a variety of enzymatic reactions, neuropeptides are synthesised in the neuronal cell body. They are derived by the enzymatic cleavage of large and generally inactive precursors or polypeptide molecules. In the cell nucleus, the gene encoding is transcribed into messenger ribonucleic acid which is translated on the surface of the rough endoplasmic reticulum into the pre-propeptide sequence. This pre-propeptide contains a signal peptide (a hydrophobic NH_2 terminal sequence) which directs the nascent protein chain into the lumen of the rough endoplasmic reticulum where protein folding and the formation of disulphide bonds occurs (Gething & Sambrook,

1992; Helenius et al, 1992). Proteolytic cleavage occurs removing the signal sequence which is then rapidly degraded. The propeptide is transported from the endoplasmic reticulum to the cis-Golgi compartment (Pfeffer & Rothman, 1987). The Golgi complex consists of three functionally distinct compartments termed *cis, medial* and *trans* which correspond to sequential cisternae in its track. The pro-peptide then moves from *cis* to *trans*-Golgi network by a series of residue fusion and budding steps. Within this network, the peptides are sorted and packaged into secretory large dense-core vesicles (LDCV) before transport to the nerve terminal. Post-translational processing occurs during this stage as enzymes present in the vesicles release the peptide from the propeptide and further modify it (as reviewed by Bean et al, 1994; Sossin et al, 1989; Lynch & Snyder, 1986). Multiple combinations of peptides derived from distinct gene products may be released separately or together from a single nerve terminal. This packaging process may relate to differences in the transcription and/or translation rates coupled with random protein aggregation in the Golgi or fusion of mature vesicles (Hekimi et al, 1991). Three models have been proposed to explain the mechanism of vesicle formation (reviewed by Pryer & Wuestechube, 1992). Neuropeptides require assistance to cross biological membranes due to their size and lipophilic nature. Thus, their release from the nerve ending occurs via exocytosis.

Rat prepro-NPY has been shown to be similar to human prepro-NPY except it is a 97 amino acid long whereas human prepro-NPY has been shown to be 98 amino acids long (Higuchi et al, 1988; Minth et al, 1984; Minth et al, 1986). The structure of rat prepro-NPY was confirmed by Allen et al, 1987 and Larhammar et al, 1987. It has been suggested from the processing of human prepro-NPY that the signal peptide is cleaved after 28 residues resulting in pro-NPY (Minth et al, 1984). The active peptide (the 36 residue NPY sequence) has been found to be followed by an amidation site where glycine is followed by 2 basic amino acids. This enables

Figure 1. General stages involved in the neuronal synthesis of neuropeptides

The DNA encoding the precursor is transcribed into mRNA in the nucleus of the synthesising neurone (1). This mRNA is then translated by ribosomes on the surface of the rough endoplasmic reticulum to form a pre-propeptide (2). The NH_2-terminal signal peptide directs the passage into the lumen of the endoplasmic reticulum, where it is subsequently removed. The propeptide is then transported to the Golgi apparatus (3) where is is packaged into secretory granules (large dense core vesicles) (4). These are transported along the axon to the nerve terminal (5). During stages (4) and (5) the propeptide is subjected to various proteolytic steps and post-translational modifications to yield one or more biologically active peptides. These peptides are then released following an appropriate stimulus into the synaptic cleft by exocytosis (6).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amidation to occur by oxidative removal of glyoxylate from glycine to form a carboxyl terminal amide (Bradbury et al, 1982). The amidation site has been shown to be followed by a 30 residue C-terminal sequence. (Higuchi et al, 1988).

Once peptides are released and bound to their receptors, they need to become inactivated to avoid a constant stimulus. Classical transmitter systems often have enzymic degrading or reuptake systems to remove the active molecule from the synaptic cleft, terminating its physiological action. There is very limited evidence for the uptake of neuropeptide ligands by cells following release in vivo (Morel et al, 1994; Beudet et al, 1994). This is consistent with the finding that peptide messengers have more prolonged actions and can function at a distance from release sites. *In vitro* studies have led to the belief that neuropeptide inactivation is carried out by peptidases in the extracellular space (as reviewed by Csuhai et al, 1995). Peptidases may be either located on the outside of the cytoplasmic membrane or secreted into the extracellular space. Post-translational modifications such as amidation and disulphide linkages are thought to assist in the longlevity of some peptide messengers, protecting them from peptidases present in the extracellular space.

NPY, PYY and pancreatic peptide all have an initial proline rich sequence which is inert to the attack of common proteinases but not specific aminopeptidases. Only a few peripheral peptidases are capable of cleaving NPY. In endothelial cells, dipeptidylpeptidase IV, a serine peptidase which is proline specific has been shown to metabolise NPY and peptide YY (Mentlein et al, 1993). Processing of NPY by this enzyme generates NPY(3-36) by removing the N-terminal dipeptide Tyr-Pro. The former fragment is a selective agonist at Y2 receptor subtype and demonstrates that post-secretory processing can modify receptor selectivity. NPY has also been found to be metabolised by endopeptidase-24.18 in rat kidney membranes (Price et al, 1991). Aminopeptidase P has been found in the kidney, lung, intestine and brain (Hooper et al, 1990). It has been found to remove the N-terminal tyrosine of NPY producing NPY(2-36) (Mediros & Turner, 1996).

Neuropeptides have been shown to be degraded by some specialised central neuropeptidases. These include aminopeptidase M, pyroglutamylaminopeptidase II, angiotensin-converting enzyme, endopeptidase 3.4.24.11, endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16. (Bauer et al, 1990; Checler, 1993; Dahms & Mentlein, 1992) which are present in the brain. The latter 2 endopeptidases have been found only to cleave 8-30 amino acid residue peptides (Dahms & Mentlein 1992) and therefore do not cleave NPY. Metabolism of NPY by endopeptidase 3.4.24.11 produces NPY(1-20) and NPY(31-36) (Mediros & Turner, 1996). In addition, NPY has been shown to be stable against degradation by angiotensin-converting enzyme (Ludwig et al, 1995) which is present in the spinal cord of the dog (Santos et al, 1988) and aminopeptidases N and W (Mediros & Turner, 1996).

1.2. GENERAL OBSERVATIONS ON THE FUNCTIONS OF NEUROPEPTIDE Y

Although the experiments in this thesis focus on NPY and the spinal cord an overview of the wide distribution of NPY in the peripheral and central nervous system will be described briefly. This peptide exerts a wide range of effects. In the central nervous system there is evidence that NPY is involved in the regulation of food intake, the control of blood pressure, may function as an endogenous anxiolytic and is involved in nociception. Peripherally NPY may play an important role in sympathetic vascular control.

1.2.1 NEUROPEPTIDE Y IN THE CENTRAL NERVOUS SYSTEM

Immunohistochemistry and radioimmunoassay techniques have demonstrated that NPY-like material is present in relatively large quantities in the brain of various mammalian species (Adrian et al, 1983; Hendry et al, 1984; Chronwall et al, 1985; Smith et al, 1985; Kohler et al, 1986; Covenas et al, 1990). NPY-like immunoreactivity (ir) has been observed in the rat brain in various hypothalamic nuclei as well as in the striatum, nucleus accumbens, amygdala, septum and throughout the cerebral cortex and hippocampus (Allen et al, 1983; Sawchenko et al, 1985; Pelletier et al, 1984; Ceccatelli et al, 1989; Aoki & Pickel, 1990). The presence of high amounts of NPY-like ir in the cortex, septum, amygdaloid body and hippocampus may suggest a role for this peptide in the limbic system (Albers & Feris, 1984; Chronwall et al, 1985; Kohler et al, 1986). In situ-hybridisation has revealed that the localisation of NPY mRNA correlates well with the distribution of ir-NPY, thus providing evidence that ir-NPY is synthesised, processed and stored in these neurones (Terenghi et al, 1987; Chan-Paley et al, 1988). This peptide has been found

co-localised with noradrenaline (NA) in a proportion of neurones in the brainstem and hypothalamic nuclei (Lundberg et al, 1980; Hunt et al, 1981a; Everitt et al, 1984; Chronwall et al, 1985; Holets et al, 1988). NPY has also been found to be co-localised with somatostatin in the cortex, hippocampus and striatum (Vincent et al, 1982; Beal et al, 1986; Chronwall et al, 1984; Kohler et al, 1987, Smith & Parent, 1986). It has been suggested that NPY has a role in the modulation of the activity of NA in certain central pathways due to their colocalisation.

Functional implications that can be derived from anatomical studies is obviously limited as these results provide no evidence of whether a co-localised peptide will be co-released. Such studies however, do highlight the areas of potential interest.

1.2.2 THE ROLE OF NPY IN FEEDING

Although the precise physiological role for NPY in the CNS is uncertain, various pharmacological and behavioural effects of centrally administered NPY have been demonstrated. Central administration of NPY has been found to stimulate food and water intake (Clarke et al, 1984; Stanley & Leibowitz, 1984; Levine & Morley, 1984).

Endogenous concentrations of NPY have been found to be highest in the paraventricular nucleus (PVN, Allen et al, 1983) and are modulated by fasting and feeding (Fuxe et al, 1983) suggesting that exogenously applied peptide engages receptors in or near the PVN to activate ingestive pathways. Injections of NPY and PYY into the paraventricular nucleus of the hypothalamus produced the most robust increase in feeding and drinking behaviours (Stanley & Leibowitz 1984, Stanley et al, 1985a & b; 1990) revealing the critical role of this hypothalamic nucleus. Morley and collegues reported that the most marked effect of microinjection was in the anterior

ventromedial nucleus (VMN), whereas Stanley and co-workers found the PVN to be the major site (Stanley et al, 1985b). As cell bodies from the arcuate nucleus send projections to the PVN (Gray & Morley, 1986) it is possible that the enhanced effect of anterior VMN micro-injections is due to an excitatory effect of NPY both on the arcuate and PVN nucleus, secondary to local diffusion. Although the drinking response observed with NPY has been reported as considerably smaller than the feeding response, it appears to be a direct consequence of this neuropeptide (Stanley & Leibowitz, 1984). The feeding effect of NPY has been shown to be highly selective for carbohydrate-rich foods (Levine et al, 1985; Stanley et al, 1986; Morley et al, 1987) and has been attenuated by intravenous infusions of glucose but not fructose (Rowland 1988). By contrast, injection of NPY into the fourth ventricle elicited a feeding response without affecting water intake (Corp et al, 1990). Other areas may also be involved in the stimulatory effects of NPY and related peptides on food and water intake but to a lesser extent (Stanley et al, 1985a & b; 1990; Morley et al, 1987). The secretion of NPY from hypothalamic neurones has been reported to be increased in food-deprived rats and reduced during the course of eating (Stanley et al 1993; Kalra et al, 1991). In agreement with this, injection of antisera to NPY (Stanley et al, 1993) or antisense oligonucleotides directed against mRNA for NPY has been found to suppress food intake in rats (Akabayshi et al, 1994).

Clinical studies have suggested that NPY & PYY are pathogenetic factors in anorexia and bulimia (Kaye et al, 1990). The concentration of NPY has been reported to be elevated in the cerebrospinal fluid of under weight anorexic patients and the concentration normalised when the patients gained weight and returned to normal menstrual cycles. Obesity in experimental animals has been found to be associated with high levels of this peptide in the hypothalamus (Kalra et al 1990; 1991; Stanley et al, 1993). A functional interaction between the neurones that contain 5-hydroxytryptamine (5-HT) and NPY has been suggested as fenfuramine, which depletes 5-HT, lowered the concentration of NPY in the hypothalamus (Rogers et al,

1991). No synergistic or additive effects of NPY and noradrenaline on food intake have been demonstrated (Levine et al, 1985). However, the effect of NPY on feeding has been reported to be attenuated by the opioid feeding pathway (Morley et al, 1983, Morley & Levine, 1985) but NPY has been shown to produce a greater and more prolonged effect than the opioid peptides.

1.2.3. NPY AS AN ENDOGENOUS ANXIOLYTIC

Central administration of NPY has been found to produce anxiolysis and sedation depending on the dose. Low doses of NPY have induced anxiolysis whereas high doses evoked sedation (Grundemar & Hakanson, 1994). Treatment for patients with anxiety disorders (such as lithium, electroconvulsive therapy and 5-HT reuptake inhibitors) have been shown to raise hypothalamic, hippocampal and frontal cortical concentrations of NPY and mRNA for NPY in rats (Weiner et al, 1992). Thus, endogenous NPY may play a role in controlling mood (Heilig, 1992; 1993; Wahlestedt et al, 1993a). Low levels of NPY in the cerebrospinal fluid are associated with loss of appetite and anxiety, and it appears that the synthesis and/or processing of NPY is altered in patients with depression (Heilig & Widerlov 1990; Widdowson et al, 1992).

1.2.4. THE ROLE OF NEUROPEPTIDE Y IN CARDIOVASCULAR CONTROL

Since NPY immunoreactivity has been found in nerves innervating peripheral and cerebral arteries and veins, in cardiac autonomic ganglia and in various

brainstem areas known to affect cardiovascular functions many studies have examined a role for NPY both centrally and peripherally.

Certain nuclei in the brainstem are important for cardiovascular reflexes, and central injection of NPY in such areas has evoked hypotension and bradycardia in the rat (Fuxe et al, 1983). Injection into the nucleus tractus solitarius of intact rats has produced significant dose-dependent, reductions of mean arterial pressure, heart and respiratory rates and tidal volume (Tseng et al, 1988; Barraco et al, 1990; Kubo & Kihara, 1990; Grundemar et al, 1991a & b).

It has also been proposed that NPY can modulate aortic baroreceptor reflexes and abolish the responsiveness to glutamate, the proposed endogenous transmitter in baroreceptor pathways (Grundemar et al, 1991a & b; Shih et al, 1992). Other possible targets for NPY in central cardiovascular regulation include the caudal ventrolateral medulla, the area postrema and the posterior hypothalamus (McAuley & Westfall, 1993).

As well as centrally, peripheral roles for NPY have been proposed. NPY is a potent vasoconstrictor agent in many arteries *in vitro* and *in vivo* and several mechanisms of action have been suggested (Fredholm et al, 1985; Lobaugh & Blackshear, 1990; Xiong et al, 1993; Xiong & Cheung, 1994). Indirect vasoconstriction via potentiation of other vasoconstrictor stimuli has been suggested as the activity of NPY on many arteries (Edvinsson et al, 1984; Lundberg et al, 1985b). Additionally, this peptide has been found to enhance NA-induced vasoconstriction (Edvinson et al, 1984; Wahlestedt et al, 1985; Pernow & Lundberg, 1988) but to inhibit the NA release evoked by nerve stimulation (Lundberg & Stjarne 1984). It has therefore been suggested that NPY could play an important role in sympathetic vascular control together with NA.

Functional studies have suggested that NPY exerts an indirect effect on the heart by modulating the release of transmitter from the autonomic nervous system. In support of this, NPY has been found to inhibit NA release in the perfused guinea-pig

heart (Wahlestedt et al, 1987) and in the isolated mouse atria (Foucart et al, 1989). Activation of presynaptic neuropeptide Y receptors has suppressed the release of both NA and NPY from sympathetic nerve terminals. Furthermore, activation of presynaptic alpha 2 adrenoreceptors by NA has been found to inhibit the release of NPY (Haass et al, 1989). In addition, NPY has been found to exert a prejunctional inhibitory effect on capsaicin-sensitive neurones in the guinea-pig left atria and inhibit the release of acetylcholine from the vagal nerve (Guiliani et al, 1989). NPY's distribution in association with the atrioventricular node and especially around coronary vessels suggests that it may play a role in coronary vasospasm (Gu et al, 1983; Potter, 1989).

High concentrations of NPY have been found in the sympathetic ganglia and in tissues such as the vas deferens, heart, atria, blood vessels and spleen all of which receive a dense sympathetic innervation (Lundberg et al, 1982; 1985 a; Hassall & Burnstock ,1984). These findings have been reported in a number of species including humans (Lundeberg et al, 1985b).

Support for a presynaptic action of NPY at sympathetic terminals has come from studies on the vas deferens. NPY has been found to be co-stored with NA and adenosine triphosphate (ATP) in noradrenergic sympathetic terminals of the mouse and human vas deferens (Adrian et al, 1984; Stjarne et al, 1986). In the whole isolated vas deferens of the rat a NPY-mediated depression of [^{3}H]-NA secretion evoked by trains of pulses has been demonstrated (Lundberg & Stjarne, 1984). However, this peptide was ineffective in inhibiting the contractile response to single stimuli in the epidydmal portion of the rat vas deferens (presumably mediated by NA release) but potently suppressed the same response evoked in the prostatic portion (Donso et al, 1988) suggesting that NPY may differentially affect NA and ATP release in epidydimal and prostatic portions of the rat vas deferens.

1.2.5. DISTRIBUTION OF NPY IN THE SPINAL CORD

NPY-like immunoreactive (ir) cell bodies & fibre terminations have been shown by immunohistochemical studies to be present within the spinal cord of many species including cat, dog, horse, marmoset, rabbit, fish and rat (Gibson et al, 1984; Marti et al, 1987; Cameron et al, 1990; Merighi et al, 1990; Roddy et al, 1990; Lewellyn-Smith et al, 1990). Although early immunohistochemical studies were performed with antisera raised against avian pancreatic peptide, these antisera probably detected mainly NPY (Hokfelt et al, 1981; Hunt et al, 1981a & b). Immunoreactive-NPY has been found in every region of the spinal grey matter (Gibson et al, 1984; Doyle & Maxwell 1994) with an especially dense concentration in laminae I-II (Gibson et al, 1981; Suburo et al, 1992; Merighi et al, 1990; DeQuidt & Emson, 1986; Sasek & Elde, 1985) with the deeper layers of dorsal horn containing lower levels. Two groups have reported that the highest concentration in the rat is in lamina I and the outer part of lamina II (Hunt et al, 1981b; Sasek & Elde, 1985), while Hokfelt's group found that immunoreactive fibres are less dense in lamina I than in lamina II (Hokfelt et al, 1981).

Immunoreactive -NPY has also been found present in intermediolateral cell columns of the thoracic and sacral segments and around the area of the central canal (Gibson et al, 1984; Krukoff, 1987; Dequidt & Emson, 1986; Llewellyn-Smith et al, 1990, Merighi et al, 1990) and to a lesser extent in the ventral horn (Allen et al, 1984; Gibson et al, 1984; Sasek & Elde, 1985; Dequidt & Emson, 1986; Krukoff, 1987; Llewellyn-Smith et al, 1990; Merighi et al, 1990; Roddy et al, 1990).

1.2.5.(a). The sources of NPY in the spinal cord

(ai) Primary afferents

Until recently, immunohistochemical studies have provided no evidence for NPY in rat dorsal root ganglion cells of primary afferents despite the fact that other peptides are readily detected. Substance P, CGRP, somatostatin and galanin are produced by many small and medium sized dorsal root ganglion neurones whose axons terminate mainly in laminae I & II of dorsal horn (Hokfelt et al, 1981; Rosenfeld et al, 1983; Skotfitsch & Jacobwitz, 1985). Immunoreactive-NPY has been found in a few dorsal root ganglion cells in cat (Gibson et al, 1984, Lindh et al, 1989) in chicken and in horse (Gibson et al, 1984). However, no cell bodies immunoreactive for NPY have been found in the rat. It was suggested that these results perhaps reflect that the levels present were below the detection limit of the immunohistochemical methods used.

(aii) Intrinsic neurones

No changes were reported in the density & distribution of NPY immunostaining after procedures known to destroy primary afferent input to the dorsal horn of rats (Hokfelt et al, 1981; Hunt et al, 1981a & b; Gibson et al, 1984). Dorsal rhizotomy did not alter the NPY like immunoreactive fibres in the lumbar dorsal horn (Hunt et al, 1981a& b) or the cervical cord (Gibson et al, 1984). This was in agreement with the lack of effect of cord section and neonatal capsaicin treatment (Hokfelt et al, 1981; Hunt et al, 1981b).

The use of colchicine in some of these studies (Hunt et al, 1981a; Sasek & Elde, 1985; DeQuidt & Emson, 1986; Krukoff, 1987) blocked axonal transport and accentuated the accumulation of neuropeptides in the cell bodies and hence assisted localisation of the latter (Norstrom et al, 1971). NPY containing cell bodies have

been detected in the superficial laminae throughout the cord and are most numerous in lamina II (Hunt et al, 1981a; Sasek & Elde, 1985; DeQuidt & Emson, 1986). In addition, NPY-containing cell bodies have been found present in the deeper layers, the region of the dorsal horn commisure and sacral parasympathetic nucleus (Hunt et al, 1981a; Sasek & Elde, 1985; Krukoff, 1987). In the pig, a few of these were found in laminae II-III without colchicine treatment (Merighi, 1990). The presence of NPY-containing cell bodies in the superficial laminae of the rat has been confirmed by a technique which detected a low concentration of peptide present in perikaryal cytoplasm without the use of colchicine (Rowan et al, 1993). Cell bodies have only been found caudal to T2 in the cat in laminae VI, IX with the highest concentration found in lamina VII (Krukoff,1987).

(aiii) Fibres of supraspinal origin

A potential source of NPY terminals in the dorsal horn are neurones with cell bodies located in the brainstem. In accordance, radioimmunoassay and immunocytochemical studies have demonstrated a widespread distribution of NPY immunoreactivity throughout the brain. In the rat, many catecholamine neurones in the lower medulla oblongata belonging to noradrenergic A1/A2 cell group and adrenergic C1/C2 cell groups contain NPY like immunoreactivity (Hokfelt et al, 1983; Everitt et al, 1984). It was suggested that on the basis of the extensive co-localisation with catecholamines in the periphery, projecting brainstem nuclei may also contain both NPY and catecholamines. They are extensively co-stored in axon terminals within the thoracic intermediolateral cell column (Blessing et al, 1987; Tseng et al, 1993). These axons are derived mainly from the rostral ventrolateral medulla (C1 catecholamine nucleus; Tseng et al, 1993) and degenerate following cervical cord transection (Hokfelt et al, 1981). However, results from retrograde tracing studies suggest that only the C1 cell group of the medulla and locus coeruleus contain

spinally projecting neurones with as few as 2% of neurones of the locus coeruleus projecting to the spinal cord containing ir-NPY (Blessing et al, 1987; Holets et al, 1988). As the locus coeruleus is a major source of noradrenergic neurones innervating the dorsal spinal cord, this would suggest that coexistence of noradrenaline and NPY in fibres in the dorsal spinal cord is limited. Double labelling studies have provided no evidence for NPY terminals that contain catecholamine in dorsal horn (Blessing, 1987) with limited co-localisation being reported in the cat (Doyle & Maxwell, 1994).

1.2.5.(b) NPY terminals-ultrastructural studies

Doyle & Maxwell reported that in the cat, the majority of structures containing NPY were axon terminals which contained mainly clear and round vesicles (Doyle & Maxwell, 1993). Occasionally large dense -core vesicles were found that were intensely immunoreactive. Almost all ir-NPY axon terminals formed synaptic specialisations such as synaptic junctions on dendrites and somata of dorsal horn neurones. Axodendritic synapses were most frequently found on medium sized dendrites. Immunoreactive terminals also formed axo-axonic synapses onto other axons in the cat substantia gelatinosa of the dorsal horn (Doyle & Maxwell, 1993). These included central axons of glomeruli which were thought to be of primary afferent origin. Such synapses have not been found in the rat (Zhang et al, 1995b).

In the lamprey, all ir-NPY material appears to originate from spinal cord cells and not from descending axons (Van Dongen et al, 1985). Immunoreactive-NPY varicose fibers have been shown to have close appositions on axons of dorsal root ganglion neurones. These fibres presumably all originate in small interneurones containing NPY localised just below the dorsal columns, since they are the only cell bodies known to provide ir-NPY fibres in the spinal cord of the lamprey (Bongianni et al, 1990).

Recently, a study in the rat using a post-embedding immunoelectron microscopy technique found low levels of ir-NPY & very low levels of ir-VIP in some primary afferent terminals in laminae I & II of rat lumbar spinal cord (Zhang et al, 1995b). This study indicated co-localisation with CGRP which is assumed to represent a marker for some sensory afferents in dorsal horn (Chung et al, 1988; Traub et al, 1989) and less frequently also with ir-galanin. They suggested that these represent unmyelinated afferents arising from small and some medium sized dorsal root ganglion neurones. Zhang & co-workers found several NPY positive nerve endings containing many synaptic vesicles, a few large dense core vesicles (LDCV) and some ir-NPY dendrites in lamina I and the outer third of lamina II. The terminals often formed synapses on NPY-negative dendrites and occasionally an NPY-positive terminal formed a glomerulus. In the inner two thirds of lamina II, ir-NPY structures were more frequent and numerous ir-NPY dendrites and dendritic-like neuronal processes with LDCV's were found. The latter frequently formed dendrodendritic synapses with NPY-negative dendrites. It has therefore been suggested that NPY transmission in the inner 2/3rds of lamina 2 appears mainly to go from ir-NPY-dendrite like processes to dendrites of unidentified neurones (Zhang et al, 1995b).

1.2.6. ELECTROPHYSIOLOGICAL STUDIES OF NEUROPEPTIDE Y

The involvement of NPY in differing functional systems may still result from a common electrophysiological mechanism. Electrophysiological studies in the periphery suggest that NPY may have an inhibitory role (Cheung & Dukkipati, 1991; Klapstein & Colmers, 1993; Cunningham et al, 1994). NPY has been shown to inhibit neurotransmitter release in enteric neurones and sympathetic neurones by inhibiting the intracellular calcium influx (Walker et al, 1988; Hirning et al, 1990; Schofield & Ikeda, 1988). In these neurones, NPY appears to inhibit selectively the N-type Ca^{2+}

channel (Hirning et al, 1990; Wiley et al, 1990; Plummer et al, 1991) which has been associated with providing Ca^{2+} influx associated with neurotransmitter release (Miller, 1990).

Many effects of NPY in the CNS can also be attributed to inhibition of transmitter release. NPY reversibly inhibits excitatory synaptic transmission from stratum radiatum to CA1 pyramidal cells as measured by a reduction in amplitude of the extracellular population spike and inhibits the excitatory postsynaptic potentials (EPSPs) evoked in these pyramidal neurones (Colmers et al, 1985; 1987; 1988; Hass et al, 1987). Thus, NPY abolishes neurotransmission in cultured hippocampal neurones by preventing glutamate release from presynaptic nerve terminals. In addition, it has been proposed that NPY has inhibitory postsynaptic actions acting at a distal dendritic site to reduce postsynaptic excitability. NPY reduces the spontaneous firing rate and hyperpolarisation in the locus coeruleus (Illes & Regenhold, 1990) and has been found to inhibit the excitatory and inhibitory synaptic potentials in 5-HT containing cells of the dorsal raphe nucleus (Kombian & Colmers, 1992). In contrast, the NMDA-induced neuronal activation by an atypical NPY receptor in C3 cells of the dorsal hippocampus has been found to be potentiated by NPY (Monnet et al, 1992 a; b).

NPY has been shown to inhibit voltage sensitive calcium currents in dorsal root ganglion neurones and this was shown to be associated with an inhibition of the depolarisation-induced (high potassium concentration) release of substance P (Walker et al, 1988). A subsequent study by Bleakman and co-workers demonstrated that the action of NPY was via the Y_2 dorsal root ganglion receptor (Bleakman et al, 1991). Supporting these in vitro studies, microinjection of NPY was reported to reduce the peripheral nerve stimulus-evoked release of substance P in the superficial dorsal horn of the cat (Duggan et al, 1991). Substance P release over the dorsal horn was reduced initially with subsequent inhibition becoming restricted to lamina I and II. The presence of ir-NPY in axons presynaptic to central boutons may be a

physiological correlate to this effect (Maxwell & Doyle, 1994). As substance P is released by noxious peripheral stimuli (Duggan et al, 1987), NPY may be involved in normal control of pain and possibly other sensory modalities.

1.2.7. NPY AND NOCICEPTION

NPY has been shown to modulate the processing of nociceptive information centrally. Intracerebroventricular (i.c.v.) administration of very low doses of NPY had antinociceptive effects in a mouse formalin procedure but induced hyperalgesia in the hot plate test (Melleado et al, 1993). Intracerebroventricular as well as intrathalamic administration of NPY to spontaneously hypertensive rats, which are hypoalgesic when compared to genetic control Wistar-Kyoto rats, induced a further elevation in nociceptive threshold in the hot plate paradigm (Merlo et al, 1990). Administered i.c.v. NPY, PYY, NPY(2-36) and the Y_1 agonist [Leu31, Pro34]NPY but not the Y_2 agonist NPY(13-36) produced dose-dependent inhibition of acetic acid-induced writhing in mice. The antinociceptive action of NPY was reversible over time and obtained at doses that did not produce detectable decreases in motor activity (Broqua et al, 1996). In contrast, lateral ventricular injection of NPY in rats did not alter the nociceptive threshold in 55°C warm-water tail flick (Heilig et al, 1992) or 54 °C hot plate procedures (Jolicoeur et al, 1991). The antinociceptive effects of i.c.v. administered NPY may be dependent on both the intensity and the modality of the painful stimulus.

Functional studies on the role of NPY in the dorsal horn have suggested that NPY has an inhibitory effect on spinal nociception. Intrathecal administration of NPY in rats has been shown to inhibit spinal nociceptive reflexes (Hua et al, 1991). Intrathecal NPY produced a dose dependent elevation in nociceptive threshold but

was not active in paw pressure test. C-terminal fragments of NPY, NPY(14-36), NPY(16-36), NPY(18-36) NPY(19-36) produced antinociceptive effects on the paw pressure tests in the rat (Hua et al, 1991). NPY was found to have mixed effects on nociceptive spinal reflexes when measured electrophysiologically (Xu et al, 1994). It had a biphasic effect in flexor reflex: excitability with facilitation at low doses and depression at high doses. Hua and co-workers did not report a hyperalgesic effect of NPY in rat nociceptive tests which may be due to a lower sensitivity of behavioural experiments. Although NPY is likely to have a modulatory role centrally, there appears to be no reports on the effects of NPY on the responses of spinal neurones to peripheral noxious stimuli following ionophoretic administration of this peptide.

1.3. NEUROPEPTIDE Y RECEPTOR TYPES

Evidence for the classification of NPY receptors by agonists will be considered along with their possible antagonists and cellular mechanisms. The initial classification will be described as well as the other subtypes which have since been cloned.

NPY receptors have been pharmacologically differentiated into several subtypes (see table 2). The distinction between Y_1 & Y_2 receptors has been based on the binding and functional potencies of a range of agonists, which are truncated or residue-substituted versions of the parent peptide (Wahlestedt et al, 1986; 1989; Fuhlendorff et al, 1990a; Michel, 1991). Based on the differential potencies of NPY and its C-terminal fragments such as NPY(13-36) or PYY(13-36) in various bioassays, Wahlestedt and co-workers first proposed this classification of receptors in 1986 & 1987. Due to the co-existence of NPY with noradrenaline within sympathetic fibres innervating blood vessels much of the original studies investigated transmission between sympathetic nerves and vascular smooth muscle. It was suggested that the Y_1 receptor was postjunctional to adrenergic nerves and that the Y_2 receptor was exclusively prejunctional at this synapse and sensitive to both NPY, PYY and the (13-36) fragment whereas the Y_1 receptor type was not sensitive to fragments (Wahlestedt et al, 1986). The Y_1 receptor has been reported to mediate an enhancement of noradrenaline-evoked vasoconstriction at the vascular sympathetic neuroeffector junction (Wahlestedt et al, 1986). Indeed, Y_1 receptors mediate most of the vascular effects although the involvement of other receptors has been proposed (Tessel et al, 1993). In contrast, a prejunctional Y_1 receptor was proposed to be present in rabbit isolated vas deferens due to the rank order of potency exhibited by NPY and some truncated analogues (Doods & Krause, 1991).

NPY Y_2 receptors in the periphery are generally considered to be localised

at prejunctional sites on sympathetic neurones, parasympathetic neurones and sensory C-fibres (Wahlestedt et al, 1986; Grundemar et al, 1990; Stjernquist & Owman, 1990; Westfall et al, 1990). These receptor types have been found to be involved in the suppression of the electrically stimulated twitch contractile response of the isolated vas deferens and inhibit acetylcholine release from the vagus nerve (Potter, 1985; Wahlestedt et al, 1986; Potter, 1989). A third type was originally proposed which did not bind peptide YY and tentatively named Y_3 (Wahlestedt et al, 1991; 1992). Y_3 receptors occur in the adrenal medulla, brainstem and heart but their effects have not been fully characterised (Balasubramaniam & Sherriff, 1990). An additional subtype has been suggested which in contrast to Y_3 binds peptide YY with high affinity (Larburthe et al, 1986). It was found to be located in the rat intestinal epithelial cells.

Other results have suggested more subtypes. Radioligand binding and receptor solubilization studies, indicated that NPY was found to bind to peptides of varying molecular weight ranging from 38 to 100kDa which suggested that NPY had more than 3 receptor subtypes (Michel, 1991). The order of binding affinity of an array of analogues in different tumour lines also suggested that more subtypes may be identified (Inui et al, 1992).

Based on the observation that NPY and C-terminal fragments were potent inhibitors of nicotine-induced catecholamine release from chromaffin cells, but PYY was ineffective, another receptor subtype has been proposed (Hexum et al, 1994). A receptor that binds all 3 peptides and called the PP-fold receptor has been found (Nata et al, 1990).

Table 2. The proposed rank order of potency of NPY agonists and related peptides at receptors Y_1-Y_3.

Studies using NPY, NPY analogues and related peptides in various model systems and cell types have demonstrated different orders of potency for the receptors Y_1,Y_2 & Y_3. Some examples of the model systems for each receptor type have been included. This table has been adapted from tables in the reviews of Michel 1991; Dumount et al, 1992; Wahlestedt & Reis 1993; Colmers & Bleakman 1994; Grundemar & Hakanson 1994; Wan & Lau 1995; Larhammar 1996.

The proposed rank order of potency of NPY agonists and related peptides at receptors Y_1-Y_3

Y_1	Y_2	Y_3
PYY≥ NPY≥ [Pro34]NPY = [Leu31Pro34]NPY >> NPY2-36 >> PP > NPY13-36 >NPY18-36	Y2 PYY ≥ NPY ≥ NPY2-36 > NPY13-36 >> [Pro34]NPY = PP	NPY≥[Pro34]NPY ≥NPY13-36 >> PYY> aPP
HEL cell line (Feth et al, 1992) **SKNMC cell line** (Fuhlendorf et al, 1990) **blood vessels** vasoconstriction (Wahlestedt et al, 1986; McAuley & Westfall 1992) **rat cerebral cortex** (Michel et al, 1990)	**SKNBE2 cell line** (Wahlestedt et al, 1990, 1992) **hippocampus** (Flood & Morley 1989) **rat vas deferens** (Wahlestedt et al, 1986, Doods & Kruas 1991) **dorsal root ganglion neurones** (Bleakman et al, 1991)	**bovine adrenal chromaffin cells** (Wahlestedt et al, 1990) **rat colon** (Cadieux et al, 1990) **rat cardiac ventricular membrane** (Balasubramaniam & Sheriff 1990)

1.3.1. NPY Y_1 RECEPTOR

Y_1 receptor requires an intact N-terminus of NPY to become fully activated. Loss of agonist potency occurs when only 1 or 2 N-terminal amino acids are eliminated or substituted (Grundemar et al, 1993a & b). Systematic d-substitutions in any of the first five amino acid residues in the N-terminal end of NPY result in a marked loss of potency at the Y_1 receptor (Boublik et al, 1990). By contrast substitutions in C-terminus, have been introduced without loss of potency. [Pro 34]NPY or similar variants have been shown to be effective agonists at Y_1 receptors (Schwartz et al, 1990; Grundemar et al, 1993b). C terminus substitutions at position 31 and at position 34 results in analogues ([Pro 34]NPY and [Leu 31, Pro 34]NPY) which have full activity at Y_1 receptors while being virtually inactive at Y_2 receptors (Grundemar et al, 1993a & b). From the results obtained using analogues, it has been suggested that the hairpin loop is not essential for binding the N and C termini of NPY molecule in close apposition (Schwartz et al, 1989;1990). However, the importance of the close steric arrangement of the N and C termini has been illustrated by the substitution from Tyr to Pro in position 20. [Pro 20]NPY has been found to break the hairpin like loop, resulting in a loss of affinity for the Y_1 receptor.

Molecular cloning of receptors has enabled characterisation of the receptor structure to be studied as well as the receptors pharmacology and tissue distribution. Using a rat forebrain cDNA library, a clone has been isolated and was subsequently identified as a Y_1 receptor (Eva et al, 1990). In addition, cDNA for human Y_1 receptor has been isolated and expressed in transfected cells. It has been confirmed that the isolated Y_1 receptor from humans is stringent in its demand on N-terminal part of the ligand (Herzog et al, 1992; Larhammar et al, 1992). Developmentally Y_1 mRNA has been detected at day 14 in the rat diencephalon and spinal cord (Jazin et al, 1993b). In the adult rat a subset of dorsal root ganglion cells express Y_1 mRNA (Zhang et al, 1994a). The distribution of the mRNA for this receptor is widespread in

the brain including the thalamus, hippocampus and cerebral cortex and hypothalamus (Eva et al, 1990; Mikkelsen & Larsen, 1992; Larsen et al, 1995).

An a-typical Y_1 receptor located prejunctionally has recently been characterised in the rabbit isolated vas deferens (Palea et al, 1995). It has also been suggested that NPY is an important mediator of feeding behaviour through a variant of Y_1 receptor (Kalra & Crowley, 1992; Stanley et al, 1992).

1.3.2. NPY Y_2 RECEPTOR

N-terminally truncated forms of NPY and peptide YY can activate Y_2 receptors (Han & Abel, 1987; Grundemar & Hakanson, 1994; Grundemar et al, 1993b). Hence, NPY (2-36) is about equipotent with NPY or PYY whereas NPY(13-36) is less potent than NPY(Wahlestedt & Reis, 1993). It has been shown that substitutions made to the centre part of the molecule did not affect the ability to bind to this receptor, suggesting that the hairpin loop of the molecule is not involved in the recognition at the Y_2 receptor per se (Grundemar et al, 1993b). The hairpin loop is not essential for binding, but it may help to create a steric conformation of the C-terminal hexapeptide amide that is favourable for Y_2 receptor recognition (Schwartz et al, 1990; Grundemar & Hakanson, 1994; Grundemar et al, 1993a). An intact C-terminal end of NPY/PYY is required for activation of the Y_2 receptor. Substitution from Gly to Pro at position 34 in [Pro 34]NPY results in a loss of affinity for the Y_2 receptor. It is well established that N-terminal fragments of NPY are inactive (Wahlestedt et al, 1986; Danger et al, 1987; Wahlestedt et al, 1987; Danho et al, 1988; MacKerrell et al, 1989; Wahlestedt et al, 1990). From studies in which amino acid residues have been substituted, it has been suggested that His 26 is important for the recognition of Y_2 receptor (Minakata et al, 1989). Not only is the amino acid sequence of the C-terminal of NPY essential but also the C-terminal amide

group since desamido-NPY and the C-terminally extended form NPY-Gly-Lys-Arg failed to activate the Y_2 receptor (Wahlestedt et al, 1986).

The Y_2 clone has only more recently been established compared to the Y_1 subtype clone which was identified in 1992. Low sequence identity has been shown between Y_1 and Y_2 clones. Y_2 mRNA has been detected in several brain areas (Gerald et al, 1995; Rose et al, 1995; Gehlert et al, 1996).

1.3.3. NPY Y_3 RECEPTOR

The Y_3 receptor recognises NPY but not peptide YY and displays a rank order of potency for NPY-related peptides that differs markedly from those of the known Y1 & Y_2 receptors (Grundemar et al, 1991b;1993). The main differences between NPY and PYY are found in the 13-23 segment, where PYY differs from NPY in 7 of 11 positions. The N-terminus of NPY appears to have some importance for the activation of this receptor, since NPY(13-36) has less affinity than the intact molecule. Moreover, like Y_1 and Y_2 receptors, Y_3 receptors require an amidated C-terminal in order to become activated. As of the NPY receptor, a change to Leu at position 31 and to Pro at position 34 of NPY has virtually no effect on the binding affinity of the Y_3 receptor. It has been suggested that NPY(18-36) acts as a specific antagonist to a newly discovered class of NPY receptors which may be present in the cardiac ventricular system (Balasubramaniam & Sherriff 1990).

A bovine receptor isolated from a locus coeruleus cDNA library was proposed as the Y_3 receptor (Rimland et al, 1991). The human clone from foetal brain (hFB22) which had 92% sequence homology with the bovine receptor have been shown not to bind NPY or PYY specifically (Jazin et al, 1993a).

1.3.4. NPY_4/PP_1 RECEPTOR

This receptor has been found to bind preferentially to PP and was called PP_1 and because of its similarity to the Y_1 receptor it was also named Y_4 (Nata et al, 1990; Ballantyne et al, 1993). PYY, NPY & PP all bind with high affinity. In addition, [Leu 31, Pro 34]NPY binds with high affinity and thus is not entirely selective for Y_1 receptors (Gregor et al, 1996; Lundell et al, 1996). Furthermore, the selectivity of this receptor seems to differ between species. PP has been reported to have a distinct receptor in dog intestinal mucosa (Gilbert et al, 1986; 1990), in rat phaeochromocytoma PC12 cells (Schwartz et al, 1987) as well as in the rat vas deferens (Jorgensen et al, 1990), rat adrenal cortex, and medulla (Whitcomb et al, 1990; 1996). The rat receptor expressed in PC12 cells has high affinity for bovine PP and exhibits very low affinity for NPY. In contrast the human receptor binds NPY with high affinity (Schwartz et al, 1987; Lundell et al, 1995).

This receptor has the highest sequence homology to the Y_1 receptor type. It has been isolated from rat (Lundell et al, 1996), mouse (Gregor et al, 1996) and human (Bard et al, 1995; Lundell et al, 1995) DNA libraries using oligonucleotide probes based upon the Y_1 sequence and polymerase chain reaction (PCR) primers. Northern hybridisation has detected mRNA for this receptor in the colon, small intestine, pancreas and prostate (Lundell et al, 1995). In addition, mRNA has been found at low levels in the brain including the hypothalamus and coronary arteries (Bard et al, 1995). Thus, the distribution of this receptor is more restricted than that of the Y_1 receptor.

1.3.5. NPY Y_5 RECEPTOR

A receptor has been isolated in the mouse with 50% sequence homology to the murine Y_1 has been named the Y_5 receptor (Weinberg et al, 1996). Although, the Y_5 receptor had equal affinity for both NPY & PYY this affinity was reduced

compared to the human Y_1 receptor. The high affinity of this receptor for PYY suggests that this receptor is different from the Y_3 receptor *In situ* hybridisation of mouse brain sections reveals expression of this receptor within discrete regions of the hypothalamus (Weinberg et al, 1996).

A rat hypothalamic cDNA encoding an NPY receptor has recently been discovered (Gerald et al, 1996). It has been suggested that this receptor is the postulated feeding receptor, its mRNA being found primarily in the CNS including the paraventricular nucleus of the hypothalamus. It has also been called Y_5 although it is not related to the mouse Y_5 receptor. This receptor has been found to be activated with equal potency by human NPY, PYY and PP. It is also activated by porcine NPY, NPY2-36 and [Leu31, Pro34] but poorly stimulated by rat PP.

1.3.6. THE SIGMA RECEPTOR

NPY and PYY have been reported to bind with strong affinities to the rat brain sigma binding sites (Roman et al, 1989; 1990). In the mouse hippocampal formation, NPY, PYY and [Leu31,Pro34] have been reported to displace approximately 35% of the specific binding of [3H](+)-SKF-10047 to sigma receptors (Bouchard et al, 1993). However, others failed to reproduce these results using the same technique (Tam & Mitchell, 1991).

Other *in vivo* studies provide evidence for the interaction of NPY with sigma receptors. In hippocampal pyramidal neurones the potentiation by NPY of NMDA-induced activation of CA3 has been suggested to occur via sigma type receptors (Monnet et al, 1992a & b). It is unlikely that NPY interacts with all of the sigma receptor subtypes, as it appears to discriminate between alpha sites labelled preferentially with (+)-[3H]SKF-10,047 and those labelled with [3H]DTG (Bouchard et al, 1993; Roman et al, 1991).

1.4. NPY ANTAGONISTS

Benextramine, a tetramine disulphide, was first synthesised by Melchiorre et al, 1979 as an irreversible adrenergic receptor antagonist. Comparison of benextramine's chemical composition to the presumed pharmacophore region of the NPY receptor has suggested that it is an antagonist of this receptor (Allen et al, 1987). Benextramine analogues have demonstrated that the benextramine binding site on NPY receptors is distinctly different from that on adrenergic receptors (Doughty et al, 1993). This nonpeptide antagonist has both *in vitro* and *in vivo* activity (Doughty et al, 1990, Tessel et al, 1993, Melchiorre et al, 1994). In the rat brain, benextramine has been shown to competitively displace a maximum of only 61% of specifically bound [3H]-NPY (Doughty et al, 1990;1992). Doughty and collegues have demonstrated that benextramine-sensitive binding sites in the rat brain were of Y_1 subtype while benextramine-insensitive sites were of the Y_2 subtype (Doughty et al, 1992). Benextramine has not been shown to discriminate between the peripheral, postsynaptic Y_1 & Y_2 receptors as it blocks the vasoconstrictive activity of both [Leu31, Pro34]NPY & NPY(13-36), in isolated rat femoral artery, with near equal potency (Tessel et al, 1993). As the constriction produced by NPY appears to be at least partially mediated by the influx of extracellular calcium, it has been suggested that benextramine blocked the NPY-induced contractions in vascular tissue by this mechanism and not through interaction with NPY receptors. In support of the suggestion that there are distinct differences between the rat brain Y_2 receptor which has low affinity for benextramine and the peripheral postsynaptic Y_2 receptor in the rat, it has been shown that benextramine almost completely abolished contractions induced by NPY, [Leu 31,Pro34]NPY and NPY13-36, in the rat isolated femoral artery (Doughty et al, 1992). Benextramines use is limited as it's effects are irreversible and it is of low potency (Doughty et al, 1993). Chaurasia and collegues synthesised new benextramine analogues as NPY functional group mimetics

(Chaurasia et al, 1994). The retramine carbon analogue N,N'-bis[6-[N-(2-napthylmethyl)amino]hexyl]-1,6-hexanediamine was equipotent with benextramine in a rat brain [3H]NPY displacement assay (Chaurasia et al, 1994). The analogues of benextramine have been reported to be selective, competitive antagonists of postsynaptic NPY receptors in the femoral artery of the rat (Chaurasia et al, 1994).

Tatemoto introduced 2 C-terminally based benzyl analogues of NPY with antagonistic properties for the Y_1 receptor subtype found in human erythroleukaemia cells (HEL). They are Ac-[3-(2,6-dichlorobenzyl)Tyr27,D-Thr32]NPY-(27-36)amide and Ac-[3-(2,6-dichlorobenzyl)Tyr27,36, D-thr32]NPY27-36 PYX1 & PYX2 respectively. Both bind to the NPY receptors in rat brain membranes which are predominantly of Y2 type (Schwartz et al, 1989). Although, PYX2 is more potent than PYX1 the peptide nature of PYX2 and its unknown stability both *in vitro* and *in vivo* it is likely that its use is limited (Wahlestedt & Reis, 1993). There have been inconsistent reports in the literature of PYX2 as a Y_1 antagonist. It has been reported to reduce the magnitude of contractions induced by NPY in both isolated uterine artery and vena cava of guinea-pig (Morris & Sasbesan, 1994). In addition, PYX2 has been reported to inhibit NPY-induced carbohydrate intake in Sprague-Dawley rats (Leibowitz et al, 1992) yet failed to inhibit food intake in obese Zucker rats via the putative Y_1 receptor (Beck et al, 1994). There is evidence that NPY is an important mediator of feeding behaviour through a variant of the Y_1 receptor (Stanley et al, 1992). Thus, it is not clear if PYX2 is an antagonist of the Y_1 receptor or if it is able to discriminate between 2 different subtypes of this receptor.

1229U91 effects has been assayed in the mesenteric arteries of the mouse and rat and in the rat vas deferens (Lew et al, 1996). This study reported that it is a competitive antagonist of smooth muscle Y_1 receptors of the mesenteric arteries. In contrast, it has some apparent agonist activity, but no detectable antagonist activity in the rat deferens assay which has previously been shown to contain prejunctional receptors of Y_2 subtype (Doods & Krause, 1991). The effect of 1229U91 is

relatively rapid in onset taking just one minute to peak and declines slightly over 5 minutes. The effect of NPY has a slower onset of at least 5 minutes and does not exhibit any significant decline. Yet, unlike a partial agonist of Y_1 it caused no rightward shift of the concentration-response curve. However, it has been suggested that the difference in time course from NPY is due to different sites of action. The difference between its reported potency in the brain (Daniels et al, 1995) and in rat vas deferens assay (Lew et al, 1996) suggests that nominally Y_2 receptors may be a heterogeneous class.

The C-terminal octapeptide NPY(18-36) has been shown to be a competitive NPY antagonist in rat cardiac ventricular membrane (Balasubramaniam & Sherriff, 1990). NPY(18-36) however, has been known to exhibit NPY agonist activity in reducing cardiac output (Boublik et al, 1990). At Y_2 like NPY receptors in various tissues, NPY(18-36) acts as a full agonist compared to NPY with a potency very similar to that of NPY and PYY (Michel et al, 1990). Therefore NPY(18-36) may be an antagonist at Y_3 like receptors, a partial agonist at Y_1 like and a full agonist at Y_2 like NPY receptors. This fragment thus serves as little use as an antagonist (Wahlestedt & Reis, 1993).

HE90481 is a potent H2 histamine receptor agonist that also binds to alpha 2-adrenoceptors (Michel & Motulsky, 1990). It blocked NPY evoked Ca^{2+} mobilisation and NPY binding to cells but with low potency (Michel & Motulsky, 1990). Its lack of specificity and low potency invalidates its use in physiological assays (Michel, 1991).

BIBP3226, SRRO107A and SR120819A, non-peptide antagonists, have been found to act at Y_1 receptors (Rudolf et al, 1994; Serradiel-Le Gal et al, 1995) but not on Y_2 or Y_3 receptors (Doods et al, 1995; Jacques et al, 1995; Lundberg & Modin, 1995). BIBP3226 has been found to reduce the duration of the vasoconstrictor response to sympathetic nerve stimulation in vascular beds of the pig (Lundberg & Modin 1995). The corresponding S-enantiomer BIBP3435 was not

active at Y_1 receptors (Rudolf et al, 1994). Transient effects of BIBP3226 on blood pressure and basal vascular conductance in spleen and mesenteric circulation have been found without major changes in heart rate. However, these changes also occurred with the S-enantiomer suggesting that this effect is unlikely to involve Y_1 receptor mechanisms. Instead it has been suggested that these effects are mediated indirectly via the charged arginine like groups (Lundberg et al, 1996).

1.5. MECHANISM OF ACTION OF NPY RECEPTORS AT A CELLULAR LEVEL

Ideally second messenger activation should be considered in relation to defined receptors. In some of the studies below however, the effects of second messengers have been linked to NPY without consideration of the receptors involved. Most of the action of NPY seem to involve a Gi/Go type guanine nucleotide binding proteins as these actions are pertussis toxin (PTX) sensitive (nodose neurones: Wiley et al, 1993, cardiac cells: Keung & Karliner, 1990, dorsal root ganglion cells: Holz et al, 1989). Biochemical analysis has revealed that dorsal root neurones contain the alpha subunits of at least two types of pertussis toxin substrates Gi_2 and Go. The latter subunit was found in the highest concentration (Ewald et al, 1989). NPY receptors in dorsal root ganglion neurones can be modulated by GTP and its stable analogues (Walker et al, 1988). The ability of NPY to inhibit calcium currents can be abolished by pertussis toxin and reconstituted by the 39-kDa alpha subunit of Go but not by 2 closely related G-proteins, Gi_1 and Gi_2 (Ewald 1988 a & b). In contrast, NPY-mediated presynaptic inhibition in the hippocampus was PTX insensitive, suggesting that tissue specific differences exist in the signal transduction pathways that couple NPY receptors to intracellular second messenger systems (Colmers & Pitman 1989). Furthermore, Y2 receptors have been shown to be coupled to multiple signal transduction pathways in the same cell type, all of which are pertussis toxin sensitive (Shigeri & Fujimoto, 1994). Whether this coupling is to activation of phospholipase C or generation of inositol triphosphates is uncertain. It has been demonstrated that NPY can stimulate the synthesis of inositol triphosphate (IP_3) and diacylglycerol in dorsal root ganglion neurones (Perney & Miller, 1989). Both NPY and bradykinin can activate IP_3 in the same cell but seem to utilise different G-proteins. Some evidence suggests a role for the enzyme protein kinase C as the ability of NPY to inhibit neuronal Ca^{2+} channels is reduced following down-regulation of

protein kinase C due to prolonged exposure to phorbol esters (Rane & Dunlap, 1986; Ewald et al, 1988a). Since mobilisation of Ca^{2+} from intracellular stores by many receptors occurs secondary to the generation of IP_3, it has been debated whether or not Ca^{2+} mobilisation of NPY receptors depends on the activation of a phospholipase C. Generation of IP3 at early time points (before or during the Ca^{2+} rise) has been convincingly demonstrated in two studies (Daniels et al, 1989; Perney & Miller, 1989). Other investigators, however, were unable to demonstrate inositol phosphate generation by NPY at early time points, even though other hormones (which were equally effective at raising intracellular Ca^{2+}) significantly stimulated inositol phosphate generation (Motulsky & Michel, 1988; Mihara et al, 1989; Lobaugh & Blackshear, 1990).

Inhibition of adenylate cyclase by NPY has been demonstrated in every tissue studied and in several cell lines, including model systems with pharmacological characteristics of Y_1 like (Michel et al, 1990), Y_2 like (Wahlestedt et al, 1990), and Y_3 like (Balasubramaniam & Sheriff, 1990) NPY receptors. Inhibition of adenylate cyclase has been demonstrated in many studies including pig spleen (Lundberg et al, 1988), neuroblastoma SK-N-MC cell line (Olasmaa et al, 1987), human erythroleukemia cells (Motulsky & Michel, 1988), rat atrial cells (Kassis et al, 1987), and the human frontal & temporal cortex (Westlind-Danielsson et al, 1987). A catalytic substitution for cAMP-dependent protein kinase A increased calcium currents in nodose neurones (Gross et al, 1990). Thus NPY receptors can couple to mobilisation of Ca^{2+} from intracellular stores via inositol phosphate dependent and independent pathways. Although the inositol phosphate-independent pathway of Ca^{2+} mobilisation is still obscure, it is clear that it does not involve lipoxygenase or cycloxygenase products of arachidonic acid or a sodium/hydrogen antiporter but instead uses a thatpsigargin sensitive store (Motulsky & Michel 1988; Aakerlund et al, 1990). NPY receptors may also stimulate inositol phosphate generation indirectly-in some cell types NPY enhances the generation induced by other hormones (Haggbald

& Fredholm, 1987; Lobaugh & Blackshear, 1990). Since some isoforms of phospholipase C are Ca^{2+} sensitive it is possible that this potentiation of inositol phosphate generation is a result of NPY-induced increases in intracellular Ca^{2+}.

(a) NPY receptors and Ca^{2+} channels

Studies investigating the mechanisms by which NPY might inhibit calcium channel activity has been complicated by lack of suitable antagonists. In addition, preparations of isolated sympathetic neurones respond in heterogeneous manner to applications of NPY (Schofield & Ikeda, 1988; Foucart et al, 1993) with modulation of Ca^{2+} channel activity being restricted to sub populations of cells. In isolated rat sensory nodose neurones opposing effects of NPY have been observed: where NPY inhibits currents via Y_2 receptors and enhances currents via Y_1 receptor activation (Wiley et al, 1993).

The mechanisms by which NPY receptors are coupled to Ca^{2+} channels has not been resolved. Bath applied NPY has been shown to be ineffective on Ca^{2+} channels recorded in cell attached patches (Hirning et al, 1990) a finding which argues in favour of a closely coupled membrane-delimited mechanism. The effects of NPY on Ca^{2+} influx through voltage-dependent Ca^{2+} channels have been examined in cultured dorsal root ganglion neurones. The inhibition of the channel has been accompanied by a slowing of the activation kinetics (McDonald et al, 1995). This effect has been described for the action of numerous other G-protein receptors on voltage gated channels (Bean, 1989). Luebke & Dunlap suggested that 2 distinct pathways for inhibition of N-type current exist: one associated with kinetic slowing which is voltage dependent, the other simply reducing current amplitude without any voltage dependence or change in current kinetics and that noradrenaline & GABA are capable of using either pathway (Luebke & Dunlap, 1994). Mobilisation of Ca^{2+} from intracellular stores was first demonstrated in HEL cells (Motulsky & Michel,

1988) and has now been found in various other cell types (Perney & Miller, 1989; Aakerlund et al, 1990; Mihara et al, 1989). Bleakman et al (1991) found that NPY inhibited the calcium influx through voltage gated Ca^{2+} channels NPY has also been effective in reducing Ca^{2+} influxes when trains of action potentials have also been used to elicit increases in intracellular Ca^{2+} (Bleakman et al, 1991). The receptor subtype mediating this inhibition of voltage dependent calcium channels appears to be of the Y_2 type by virtue of its response to C terminal fragments (Bleakman et al, 1991). In acutely isolated vagal afferent neurones dissociated from rat nodose ganglion, NPY at maximal concentration blocked about 30% of the peak current ganglion via Y_2 receptors. In approximately 5% of these neurones, application of NPY increased the influx of Ca^{2+} through voltage dependent Ca^{2+} channels by about 20%. This facilitation has been reported to result from the activation of Y_1 receptors (Wiley et al, 1993).

NPY has been shown to inhibit Ca^{2+} currents in sympathetic cells (Plummer et al, 1991). Although the Y_2 receptor has been shown to mediate inhibition of noradrenaline release from sympathetic terminals in intact preparations (Whalestedt & Reis 1993), it appears that inhibition of voltage sensitive calcium channels in acutely isolated superior ganglion cells is mediated by a different receptor (Foucart et al, 1993). The agonist profile resembles that of the Y_3 receptor (Balasubramaniam & Sherriff, 1990; Grundemar et al, 1991b).

In the neurones of the bullfrog paravertebral sympathetic ganglia, NPY, like noradrenaline activates, an inwardly rectifying potassium current (Zidichouski et al, 1990). However, NPY has been found to potentiate but not activate the inwardly rectifying potassium current produced by activation of the alpha 2 adrenoreceptors in locus coeruleus neurones (McEnery et al, 1994).

In SH-SY5Y cells the effects of NPY were additive with nifedipine suggesting that NPY predominantly inhibits N-type Ca^{2+} channels. This finding is in agreement with the results obtained with NPY's presynaptic action in sympathetic

neurones which were co-cultured with atrial myocytes (Toth et al, 1993). Similarly, NPY has been shown to inhibit somatic N type channels in isolated sympathetic and sensory neurones via Y2 receptor activation (Foucart et al, 1993; Wiley et al, 1993). Inhibition of N-type Ca^{2+} channels by NPY has been studied electrophysiologically in various neuronal preparations including dorsal root ganglion neurones (Walker et al, 1988; Perney et al, 1989), rat vagal afferent neurones (Wiley et al, 1990) and cultured rat myenteric neurones (Hirning et al, 1990). These studies have also demonstrated that inhibition is mediated via Go and is not accompanied by inhibition of L or T-type Ca^{2+}channels (Wiley et al, 1990; Hirning et al, 1990). Inhibition of N-type Ca^{2+} channels in neuronal preparations may be the mechanism for presynaptic inhibition of transmitter release.

Evidence that L type Ca^{2+} channels are activated by NPY is indirect and comes mainly from functional studies on vascular smooth muscle. Such studies demonstrate that NPY-stimulated direct and indirect (*i.e.* potentiation of noradrenaline response) vasoconstriction *in vitro* and *in vivo* is sensitive to dihydropyridine-type Ca^{2+} entry blockers (Lundberg et al, 1985b & 1988).

CHAPTER 2: Introduction to Neuropathic Pain

2. NEUROPATHIC PAIN

This second part of the introduction focuses on neuropathic pain. It begins with a brief historical description of neuropathic pain in humans followed by a description of the associated animal models developed. The role of the sympathetic system after nerve injury is discussed as well as the changes which occur peripherally and centrally in rats with a partial nerve injury produced using the Bennett & Xie method.

2.1. COMPLEX REGIONAL PAIN SYNDROMES-CAUSALGIA

After the American civil war, causalgia was first described by Mitchell as a painful syndrome which sometimes develops after peripheral nerve injury associated with penetrating wounds but not involving a complete transection. Causalgia is characterised by a rapid onset (usually within hours of insult) of spontaneous constant burning pain in addition to hyperalgesia (increased sensitivity to noxious stimuli) and allodynia, pain response to low threshold stimuli which is normally innocuous, (Mitchell, 1872; Livingston, 1943, Sunderland & Kelly, 1948). The constant burning pain has been found to be exacerbated by several factors such as temperature change, movement of the involved limb, visual and auditory stimuli and emotional disturbances (Bonica et al, 1970; Loh & Nathan, 1978; Merskey, 1986). The pain has been reported to sometimes spread beyond the cutaneous distribution of the damaged nerve (Merskey, 1986; Proccacci & Maresca, 1987; Rabin & Anderson, 1985). In addition, the pain may appear in mirror image sites on the contralateral intact limb (Rabin & Anderson, 1985; Mitchell, 1872). This syndrome has been well documented especially in military populations (Mitchell 1864; Livingston 1943, Sunderland & Kelly, 1948; Richards, 1967; Sunderland, 1976; Bonica, 1979). In addition to the

above syndrome others have used different criteria in identifying cases of causalgia (for review see Schott, 1986). Patients with a similar pain syndrome without evidence of nerve injury have been reported as mimocausalgia (Patman et al, 1973; Thompson & Patman, 1975) and minor causalgia (Hardy et al, 1958; Wirth & Rutherford, 1970).

In 1953 Bonica reserved the term causalgia for persistent burning pain that follows traumatic injury of a major peripheral nerve and later reported that this pain may be exacerbated by sympathetic arousal or tactile stimulation (Bonica, 1953; 1970). In 1979 the syndrome was redefined by the International Association for the Study of Pain (IASP) as a syndrome of sustained burning pain after a traumatic nerve lesion combined with vasomotor and sudomotor dysfunction and later trophic changes. This pain was often accompanied by hyperpathia (delay, over reaction and after sensation to stimulus, especially repetitive stimuli) and allodynia (IASP 1979, Merskey, 1982). Tahmoush reported that this syndrome occurred after a wide spectrum of injuries in both civilian and military populations, either with or without clinical evidence of nerve damage of vasomotor and sudomotor dysfunction and trophic skin changes (Tahmoush, 1981). He suggested that the autonomic disturbances had been overemphasised in the IASP definition and thus suggested an alternative set of criteria that were necessary to define causalgia. Tahmoush suggested that within weeks prior to the onset of pain a traumatic event occurs proximal to the painful area. The presence of hyperalgesia, allodynia and continuous burning pain distal to the site of injury were required to define the syndrome as causalgia.

In some patients with trauma-related continuous pain syndromes, dystrophic changes in a limb are prominent. Evans (1946) described the global term reflex sympathetic dystrophy (RSD) to describe clinical conditions in which pain was likely to be maintained by the ongoing activity of the sympathetic nervous system. The term algodystrophy has also sometimes been used to describe this syndrome. Minor tissue trauma and fractures and non-traumatic nerve lesions were among the precipitating

events. The term RSD has generally been used when the condition was not associated with obvious peripheral nerve trauma. The changes associated with this syndrome have been found to occur distally in the affected extremity showing a triad of autonomic, motor and sensory symptoms with a glove- or sock-like distribution being reported (Blumberg, 1991; Blumberg & Janig, 1993; Kurvers et al, 1995). Patients have some or all of the following symptoms: spontaneous burning pain, hyperalgesia , hyperpathia (delayed and exaggerated painful response to stimuli), dystrophic changes in skin and deep somatic tissues and a conspicuous abnormal neural regulation of sympathetic reflexes supplying blood vessels and sweat glands (Rizzi et al, 1984). Similarities between causalgia and RSD have frequently been described (Evans, 1946; Bonica 1953).

The use of sympathetic blocks also led to the idea that some patients have sympathetically maintained pain (SMP) and others sympathetically independent pain (SIP) (Roberts & Elardo, 1985). The term sympathetically maintained pain was introduced by Roberts (1986) and subsequently used by others (Campbell et al, 1992; Frost et al, 1988; Meyer et al, 1992). SMP may be a feature of several types of painful disorders thus it may accompany a wide spectrum of disorders. The principal symptoms are spontaneous pain and mechanical and cold allodynia within the zone of the lesioned nerve (Blumberg & Janig, 1993; Frost et al, 1988; Meyer et al, 1992; Tredde et al, 1992; Torebjork et al, 1995). The definition of SMP is entirely pragmatic, depending on the outcome of regional blocks. It has been reported that sympathetically maintained pain is a phenomenon that appears to be inconsistent and variable over time, such that at one observation, a given patient may have pain that is partially or wholly sympathetically maintained but at another (usually later) time, the pain may be entirely sympathetically independent. In addition some authors have reported that a series of blocks may be necessary to produce pain relief (Wynn Parry & Withrington, 1984) which creates a practical problem in the diagnosis of SIP.

In 1995 the IASP redefined causalgia, RSD and SMP. The classification was based upon the signs and symptoms as the underlying mechanisms for these disorders remain unclear (Stanton-Hicks et al, 1995). Causalgia and RSD were termed as complex-regional pain syndromes (CRPS) with specific criteria determining the type of syndrome. The overall term, CRPS, infers that regional pain and sensory changes following a noxious event must be present. The name is intended to be self explanatory. Complex was used to denote the varied clinical phenomena found in addition to pain. It is usually the distal part of a limb that is affected although the symptoms have also been reported on the torso or face and may spread to other areas (Bentley &Hammeroff, 1980) thus the term regional has been used. Pain is the cardinal symptom for this syndrome.

Type I corresponds to the most common disorder previously known as RSD and attempts to describe the symptoms of the typical patient without a definable nerve lesion whereas type II, formerly called causalgia, refers to cases where a definable nerve lesion is present. The frequent accompaniments of altered vasomotor, sudomotor and trophic states were not included as diagnostic prerequisites since no constant relationship between these states and the pain has been established. CRPS type 1 (RSD) develops after an initiating event. It typically has deep, diffuse pain which may respond to sympathetic block. Variable etiological factors can include repetitive minor trauma, stoke, immobilisation, myocardial infarction and over-strain (Askey, 1941; Steinbrocker et al, 1948; Moskowitz et al, 1958; Patman et al, 1973; Low & Nathan, 1978). Both syndromes report pain that is disproportionate to the inciting event either in the form of spontaneous pain and/or allodynia and hyperalgesia which may extend beyond the territory of a single peripheral nerve. There is evidence at some time (not necessarily at the time of diagnosis) of edema, changes in skin blood flow or abnormal sudomotor activity in the region of pain. CRPS type II (causalgia) develops after nerve injury and is intended to include patients fitting the classical

description of causalgia (Richards, 1967; Bonica, 1970; 1990). These patients have a partial lesion which is acutely followed by pain within the zone of the lesioned nerve.

This report emphasised that SMP may be a feature of several types of painful conditions and is not as essential requirement of any one condition. It is pain that is maintained by sympathetic efferent innervation or by circulating catecholamine. It was suggested that a patient may have a portion of the pain which was sympathetically maintained and a portion sympathetically independent. It can be associated with a number of diseases and a positive response to a sympathetic block should neither be a factor in the nomenclature of the disease nor define the disease.

2.2. DIFFERENT MODELS OF NEUROPATHIC PAIN

Peripheral nerve transection has been extensively studied experimentally but it does not produce the signs and symptoms associated with clinical nerve damage since there is complete deafferentation. Therefore models have been developed which more closely resembled the human syndromes associated with nerve injury. The Bennett & Xie model was the first animal model of this type and will be described in the greatest detail in the following sections as this was used in the present studies. The initial study for each of these these partial nerve injury models is desribed below.

2.2.1. THE BENNETT & XIE MODEL

Bennett & Xie described this procedure in 1988. The following description is taken from this publication. Adult male Sprague Dawley rats were anaesthetised with sodium pentobarbital and the common sciatic nerve exposed at the middle of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatic nerve's trifurcation, the nerve was freed of adhering tissue and 4 ligatures (4.0 chromic catgut) were tied loosely around the nerve with approximately 1mm spacing. The ligatures were tied so that the diameter of the nerve was found to be just barely constricted when viewed with 40x magnification. Sham operations were performed on the opposite side of each animal.

Pain-related behaviours ipsilateral to the sciatic ligation were found to occur in most of the rats by 6-8 days after surgery and became maximal during the second postoperative week (12-15 days). The results obtained by this initial study are described in more detail below.

2.2.1.(a) Posture and gait

The gait of the rat was most variable during the initial week postoperatively. The affected paw was everted with only the medial edge of the foot touching the floor. Toes were held together and were ventroflexed. Rats were often seen to suddenly raise the affected hind paw quickly from the floor and hold it in a protected position next to the flank while standing or sitting. Bennet & Xie (1988) suggested that this sudden action was due to sudden pain. In addition, the rats walked with a limp which was most prominent during the first few postoperative days and became much less noticeable after 4-6 weeks. About 70% of the rats exhibited autotomy (self-mutilation) which began on the first postoperative day and was most common during the first 10 days postoperatively. Extensive autotomy was rare. In most cases autotomy began with gnawing of the claw tips and proceeded until the root claw was bloodied. Autotomy was not evident on the hind paw on the sham-operated side.

2.2.1.(b) Mechanical allodynia

Mechanical thresholds were determined using a Randall-Selitto analgesymeter. Calibrated pressure of gradually increasing intensity was applied to the middle of the hind paw dorsum between the second and third or third and fourth metatarsals until the rat withdrew the hindpaw. Placement was varied slightly between trials and the stimulus intensity was recorded as grams of force. Hind paws were tested alternatively at 3-4 min intervals until 3 measurements were obtained for each side. A difference score was calculated by subtracting the average of the control side from the average of the ligated side. Difference scores between the right and left hind paws were determined before surgery and on postoperative day 19. The preoperative withdrawal threshold and the preoperative mean difference scores for the control group did not differ significantly from those obtained for the ligated animals. Postoperative mean difference scores for ligated animals and separate sham operated

animals were not significantly different from each other. Thus evidence of mechanical allodynia was not obtained in this initial study.

Evidence of mechanical allodynia and hyperalgesia however was found in the study of Attal et al (1990). They used a modified version of the analgesymeter to determine the vocalisation threshold to paw pressure. Until day five the lesioned paw was hypoalgesic as indicated by a significant increase in thresholds for vocalisation. By two weeks after surgery the mean vocalisation threshold of the ligated paw was approximately 66% of the preoperative control reflecting a significant allodynia. The threshold for vocalisation was also significantly reduced in the opposite paw, the sham operated paw, compared to the preoperative control values at this time. The mechanical allodynia of the ipsilateral hind paw was thus maximal during the second week postinjury after which time it began to decrease until recovery by week 8.

Attal and co-workers used a pair of graduated smooth serrated forceps to pinch the middle of the plantar hind paw. The onset of hyperalgesia in the ligated hind paw appeared to be variable during the first five days postoperatively when some rats were hypoalgesic. Two weeks after surgery, the majority of rats had a maximal reaction to pinch. This hyperalgesia declined gradually until complete recovery at week 12. In contrast, no changes occurred in the sham operated paw after surgery.

2.2.1.(c) Thermal allodynia

Normally innocuous thermal stimuli were applied to the hind paw after surgery to detect whether allodynia was present after surgery. Rats were exposed for 20 minutes to a floor that was warmed by a 30°C bath or chilled by a cold water bath at 4°C. The behaviour of the animal was noted and an event recorder was used to measure the time that either hind paw was held above the floor while the animal was sitting or standing. Occasionally the rats stood with the affected hind paw elevated above the floor for 1-5 seconds. Thirty days after surgery, the cumulative duration of

such events averaged 30 seconds for the 20 minute observation period Paw withdrawals were more frequent and often occurred in a series of 3-4. The average frequency of hind paw withdrawals on the nerve-damaged side increased 5-fold and the average cumulative duration of the hind paw withdrawals increased 2-fold. The cold stimulus frequently did not elicit a response in the hindpaws of control animals.

2.2.1.(d) Thermal hyperalgesia

To behaviourally test the response to noxious heat, rats were placed beneath an inverted clear plastic cage upon a glass floor. A heat source placed at 90° to the hind paw was positioned such that the proximal 1/2 of the plantar surface was irradiated. Stimulus onset activated a timer that was controlled by a photocell positioned to receive light reflected from the hind paw. By interrupting the photocell's light the hind paw withdrawal reflex automatically stopped the timer. The hind paws were tested alternately with 5 minute measurements taken for each hind paw in each test session. The 5 latencies per side were averaged and a difference score computed by subtracting the average latency of the control side from the average latency of the ligated side. The results were expressed as difference scores and result from the ligated and sham operated animals were compared.

A single heat test session during the first 5 postoperative days evoked severe edema in the hindpaw on the ligated side in the majority of rats but not in the sham operated side. Although the onset of hyperalgesia was earlier than day 5 for some animals, tests were performed after postoperative day 5 to avoid this edema. Eighty five percent of the rats were hyperalgesic (as defined by a negative mean difference score that was more than one standard deviation below the mean of the normal population). The remaining 15% of animals were hyperalgesic by days 8-10. Hyperalgesic responses to noxious radiant heat were evident on the second post operative day and lasted for over 2 months.

2.2.1.(e) Temperature changes in the hind paws

The temperature of the plantar surface of the hind paw was measured by placing it on a small plate made of thin copper which had a thermistor attached at the site where the paw was placed. The hind paw was held against the plate such that the plate was covered completely by the part of the sole that lies proximal to the plantar pads. Stable temperature readings were obtained in 30-60 seconds and measured to the nearest 0.1°C. Only 1 out of 30 normal rats had a temperature difference greater than ±1.0°C when the temperature of the right paw was subtracted from the temperature of the left paw. In contrast, temperature differences greater than 1°C were found more frequently when the hind paws in the nerve injured animals were compared. The hind paw on the injured side was found to be either abnormally cool or warm relative to normal control values. Therefore, the mean temperature difference of the nerve damaged group was approximately zero and thus not significantly different from the mean of the normal group.

2.2.1.(f) Chemical hyperalgesia

Mustard oil was applied to the distal 2/3rds of the dorsal hind paw to demonstrate hyperalgesia to a noxious chemical stimulus. Notes were made of the animals behaviour and a stopwatch was used to measure the latency of the initial reaction and the duration of time that the hind paw was held above the floor during a 5 minute observation period. Prior to nerve injury mustard oil evoked a stereotyped series of responses. After an average latency of 11 seconds, the rats rapidly shook the treated hindpaw rapidly during the next 15-20 seconds. The shakes were too rapid to count and usually occurred as the rat walked. This was followed by grooming the paw which only lasted a few seconds probably because the mustard oil tasted unpleasant. Postoperative responses from the hind paw on the sham-operated side were identical to the preoperative responses but the responses of the ligated side were

clearly abnormal. The rapid shaking and grooming was similar but the hindpaw was elevated above the floor for over 30 seconds at a time. When the paw returned to the floor another prolonged withdrawal was often evoked immediately.

2.2.2. THE MODEL OF KIM AND CHUNG

In this procedure the lumbar (L) spinal nerves L5 and L6 or the L5 spinal nerve alone of one side of the rat were tightly ligated with 3.0 silk thread. The same procedure was carried out on the opposite side of the animal without nerve ligation. The results obtained by the initial study by Kim and Chung (1992) are described below.

2.2.2.(a) Posture and gait

Postoperatively, the paw of the rat was everted on the injured side and the toes held together. Sixteen weeks later (at the end of the study) this deformity was still present. The hind paw on the operated side was held elevated in the air in a protected position for 6 weeks. Observations of the general behaviour of the rat suggested that spontaneous pain had developed in the hind paw after the surgical procedure. The rat sometimes suddenly licked the operated hind paw which was followed by immobility in the absence of external stimuli. In addition, licking of toes and pulling the nails with the mouth occurred although signs of autotomy were absent. The licking behaviour may be correlated with that of the human neuropathic patient who attempts to relieve pain by wetting the painful extremity with water (Richards, 1967; Tahmoush, 1983).

2.2.2.(b) Mechanical allodynia

The rats were placed on a metal mesh floor and mechanical stimuli were applied from underneath the animal with 6 different Von Frey hairs to the plantar surface of the foot. The occurrence of foot withdrawal was expressed as a frequency of response. From twelve to twenty hours after surgery the rats began to respond to mechanical stimulation by the weakest Von Frey hair (8.4mN) which produced no response prior to injury. The strongest force (186.7mN) produced no response in shams but a response in both the ipsilateral hind paw from day one and contralateral hind paw from postoperative day 21. This sensitivity on both paws was still present at 16 weeks which was the end of the study period.

2.2.2.(c) Thermal hyperalgesia

The latency of foot withdrawal to noxious heat stimuli was measured using the method described by Bennett & Xie (1988). On the side ipsilateral to the nerve injury, the withdrawal latency to heat was shorter and the behavioural response to noxious heat stimuli became exaggerated. Licking the paw after stimulation was frequently observed. This model produced a long-lasting hyperalgesia to noxious heat which was significant from day 3 until postoperative week 5. After this time, the difference scores were not significantly different from that of the unoperated control group. These results suggested that the peripheral nerve injury induced thermal hyperalgesia in these rats.

2.2.3. THE MODEL OF SELTZER AND SHIR

This model was described in 1990 by Seltzer et al, 1990. Under ether anaesthesia the sciatic nerve was exposed at high thigh level. Silicon-treated suture

(8-0) was inserted into the nerve and tightly ligated around 1/3rd to 1/2 of the sciatic nerve thickness leaving the remainder unaltered. Sham operations were performed in separate animals.

2.2.3.(a) Posture and gait

After surgery, the paw was guarded by holding elevated in the air and when placed on the floor the two lateral toes were flexed. This deformity was found to last for several weeks and when the rats were encouraged to walk no limping was noticed.

2.2.3.(b) Mechanical allodynia

Mechanical allodynia was determined using Von Frey hairs in ascending order of force. A quick flick of the paw indicated that threshold had been reached. One hour after injury the average thresholds in the injured paw and in contralateral intact paw decreased. This decrease was more marked in the injured side. These bilateral changes remained during the follow up period which extended to 54 days postoperatively.

2.2.3.(c) Mechanical hyperalgesia

A single prick was given at the midplantar area using a sharply pointed plastic rod and the behavioural response noted. Pin-prick elicited exaggerated responses in the paw ipsilateral to the nerve injury. Fifty four percent of the responses to pin-prick were exaggerated by the second postoperative day. The proportion then decreased to 30% at which it remained for the next 5 weeks. Exaggerated responses to this stimulus were also obtained for the contralateral intact paw, but were less frequent than those obtained for the operated side.

2.2.3.(d) Thermal allodynia

The withdrawal threshold to heat was determined by beaming short pulses of infra-red radiation from a CO_2 laser at the mid-plantar area of each paw and averaging the values obtained at 3 different locations. Thresholds were measured by gradually increasing the duration of the pulses until the rat responded with a paw flick. The threshold for a paw was calculated as the average of the threshold determined at 3 locations per paw. In sham operated animals, the withdrawal threshold to noxious heat pulses did not change postoperatively. However, a bilateral decrease in threshold was obtained in animals with a partial nerve injury. This was a rapid (present 1 hour after surgery) and long-lasting decrease in the withdrawal threshold to heat stimuli. A suprathreshold heat stimulus produced a quick flick and brief licking of the hindpaw in sham animals. In addition to a more robust flick when the injured paw was stimulated the behavioural response was exaggerated with the animal attempting to escape. As the actions of the animal were variable the duration of the response was quantified. The peak ipsilateral increase occurred during the first week postoperatively, thereafter it declined to a plateau which was still more than double the preoperative value, or that of the contralateral intact hindpaw. When studied 7 months postoperatively, the response duration at the right hind paw was still twice that of the left side.

2.2.3.(e) Thermal hyperalgesia

Three CO_2 laser heat pulses of noxious but non-tissue damaging intensity were aimed at the mid-plantar area of each foot, alternating between the two hind paws. The time that lapsed between lifting the paw until replacing it on the floor was recorded.

Decreased withdrawal thresholds to CO_2 laser pulses appeared in 97% of partially injured rats, both in the right and left paws. Increased response duration of a

suprathreshold heat pulse was detected in the right hind paw in 78% and in the left hind paw in 11% of the partially injured rats.

2.2.3.(f) Cold allodynia

To test the behavioural response to a cold stimulus a metal plate with a surface temperature of 5 ±0.2 C was used and the time the paw was in the air measured. When the rats were placed on a 5°C cold plate for 5 minutes, at day 14 postoperatively, unoperated paws were withdrawn significantly more than operated paws.

2.2.4. DELEO'S MODEL

Cryoanalgesia, the technique of freezing nerves, was first used clinically for the treatment of postoperative and chronic pain. Paradoxically, this same technique has been found to produce characteristics in a rat model suggestive of neuropathic pain (DeLeo et al, 1994). Freeze lesions temporarily disrupt normal functioning but do not induce endoneurial structural damage. This peripheral nerve lesion has been produced in the rat by applying a cryoprobe cooled to -60°C in a 30-5-30 second freeze-thaw-freeze cycle proximal to the sciatic nerve trifurcation. In addition, the contralateral nerve was exposed for the same time period but not cryolesioned serving as a sham-surgery control.

2.2.4.(a) Posture and gait

Immediately following recovery from anaesthesia the rats were found to hold the injured paw above the floor. The toes were closed and ventroflexed and the

sole of the foot was turned up medially. This postural sign was evident until approximately 14-16 days postoperatively. The spontaneous nociceptive behaviour of the rat was assessed as previously described by Hayes et al (1992). Rats were observed for 5 minutes and the time course of these behaviours recorded. The animals were studied for signs of elevation of the hind paw, licking or scratching of the hind paw or placement of only the medial edge of the heel on the surface.

Autotomy was present in this model from the first day after surgery. The average onset occurred by 4 days postoperatively and significantly increased in severity between 10-14 days. By 3 weeks the presence of autotomy had disappeared and substantial healing of the affected paw was present.

2.2.4.(b) Mechanical allodynia

To determine mechanical allodynia Von Frey hairs were used. The gauge of probe that elicited foot withdrawal was recorded as the sensitivity threshold for the paw on that day. After injury until day 15 the tactile sensitivity in the operated paw was significantly suppressed but there was no change in the sensitivity of the control paw. At 15 days postoperatively tactile sensitivity in the operated paw returned to normal, however mechanical allodynia was evident in contralateral paw at this time. By 26 days postoperatively mechanical allodynia was present in both paws and persisted until 42 days.

2.2.4.(c) Thermal allodynia

Hindpaw withdrawal reflexes were measured as previously described. Immediately after the nerve lesion, paw withdrawal to radiant heat was reduced indicating that the affected paws were hypoesthetic. Rats did not withdraw the affected limb from a radiant heat until postoperative day 7. By the beginning of the

3rd postoperative week, 80% of the rats had returned to their baseline latency in response to the noxious heat stimulus.

Summary

Although these models differ in duration and onset times they produce comparable behavioural changes including allodynia and hyperalgesia to both mechanical and thermal stimuli. The model of DeLeo et al (1994) has been included as it demonstrated that freezing the nerve produces some behavioural changes (mechanical and thermal allodynia) similar to different types of ligation of the peripheral or spinal nerve. This model also results in severe autotomy which was also present in the model of Bennett and Xie but was not extensive. These models may be analogous to the clinical conditions associated with peripheral nerve injury now termed the complex regional pain syndromes type I & II (Stanton-Hicks et al, 1995). The ligation models have also been found to respond to sympathectomy. Although, this was not described when these models were initially presented, the sympathetic role is worth further discussion as it may help explain why these behavioural changes occur (see section 2.5).

2.3. NERVE HISTOLOGY OF THE SCIATIC NERVE-CHANGES AFTER NERVE INJURY

Bennett and Xie found constrictions beneath each of the 4 ligatures and that the diameter of the nerve had reduced by 25-75% at the sites of constriction by the first postoperative day (Bennett & Xie, 1988). Adjacent constrictions have been shown to merge 2-3 days postoperatively. At this time, almost 70% of the nerves examined had a distinct swelling just proximal to the most proximal constriction but a caudal swelling was rare (Bennett & Xie, 1988). By 3 days a neuroma had formed as well as a dense mass of connective tissue at the ligated region that had adhered to adjacent muscle (Bennett & Xie, 1988; Coggeshall et al, 1993). This neuroma has been characterised by a disrupted perineurium and numerous growth cones (Sunderland, 1988). Nerves examined 1-3 weeks after surgery had either 2 constrictions or a region with a broad reduction in diameter which resulted from the merging of adjacent constrictions. The majority of nerves had a pronounced swelling just proximal to the constricted region and most had a distinct, but smaller, swelling distally at 1-3 weeks. Although the ligatures had appeared to lose their continuity at 10-14 days postoperatively, suture debris has been found in the connective tissue during the third postoperative week (Bennett & Xie, 1988; Guilbaud et al, 1993). Two to four months after surgery, the connective tissue capsule had been resorbed and the proximal and distal swellings had gone although the ligated region remained thinner than normal (Bennett & Xie, 1988; Coggeshall et al, 1993).

2.3.1. THE NATURE OF THE NERVE INJURY

It has been suggested from recent results that several local factors may be critical for the development of the behaviours characteristic of the Bennett & Xie model (Maves et al, 1993; Myers et al, 1993; Clatworthy et al, 1995). These factors include peri-axonal inflammation, an acidic milieu around the nerve, ischemia and endoneurial fluid pressure. The evidence supporting these factors is described below.

2.3.1.(a) Peri-axonal inflammation

The role of peri-axonal inflammation in the development of guarding behaviour and thermal hyperalgesia in the Bennett & Xie model of neuropathic pain has been investigated (Clatworthy et al, 1995). The sciatic nerve was ligated with silk, plain or chromic gut and all nerves were found encapsulated by scar tissue by day 20 or 30 postoperatively although nerves ligated with silk had less encapsulation. In agreement with this result, the use of both plain and chromic gut sutures in human surgery has been shown to incite a local tissue inflammatory response (Stewart et al, 1990). The failure of silk to produce hyperalgesia in this loose ligation model provides indirect evidence in support of the hypothesis that the development of hyperalgesia associated with loose ligation depends upon a local inflammatory response as silk has been found only to produce a small inflammatory response (Maves et al, 1993).

The presence of a foreign material (the ligature) will initiate a localised inflammatory reaction resulting in a granuloma close to the ligated peripheral nerve. Cytokines released from inflammatory cells form part of the cascade of chemical changes in the microenvironment. It has been suggested that these changes may lead to excitation or sensitisation of afferent endings (Cunha et al, 1991; Maves et al, 1993; Sommer et al, 1993). Clatworthy and co-workers investigated the role of peri-

axonal inflammation in the development of thermal hyperalgesia and guarding behaviour by using cotton sutures soaked in Freunds complete adjuvant prior to ligation (Clatworthy et al, 1995). This study suggested that the presence of inflammation close to the peripheral nerves is critical for the development of hyperalgesia in this model.

It has been shown that daily intraperitoneal injections of dexamethasone prior to and after ligation reduced the inflammatory response induced by the sutures and prevented the development of guarding behaviour and thermal hyperalgesia (Clatworthy et al, 1995). The numerous inflammatory cells (macrophages, neutrophils, multi-nucleated giant cells) which have been found surrounding the normal nerve were found to be absent from the nerve of dexamethasone-treated animals.

2.3.1.(b) Acidic milieu and the chemicals in chromic gut

The possibility that a chemical component of chromic gut sutures interacts with the sciatic or sympathetic nerves to produce pain has been investigated (Maves et al, 1993). Alterations in the rats posture associated with the Bennett & Xie model have been demonstrated when chromic gut is placed alongside but not around the nerve (Maves et al, 1993). The chemicals associated with chromic gut may act indirectly by inducing or enhancing an inflammatory response around the nerve (Clatworthy et al, 1995). Plain gut sutures appear to produce a similar inflammatory reaction to that produced by chromic gut sutures, but only chromic gut ligatures produce behavioural changes associated with neuropathy. It has been suggested that the chromic gut results in a change in the chemical milieu around the nerve or the closely associated sympathetic nerves. Chromic gut sutures are plain gut sutures to which chromic salts and pyrogallol have been added.

Pyrogallol is an inhibitor of catechol-o-methyltransferase (COMT) and

endothelium-derived relaxing factor (EDRF)/nitric oxide production. Inhibition of EDRF/NO may result in a number of vascular and neuronal changes. Altered sympathetic function may result from inhibition of COMT because catecholamines are rapidly inactivated by this enzyme even though the principle pathway for noradrenaline termination is reuptake into nerve terminals. Inhibition of EDRF/NO may result in a number of vascular and neuronal changes. Preliminary results from Maves and co-workers revealed that a continuous infusion of pyrogallol directly on the sciatic nerve for 7 days produced a marked thermal hyperalgesia and postural change identical to the loose ligation model from day 2 until day 20.

As an alternative mechanism of the effects of chromic catgut, it has been suggested that chemicals released from chromic ligatures causes a reduction in local pH producing activation of capsaicin sensitive afferents in the sciatic nerve (Geppetti et al, 1990; 1991).

2.3.1.(c) Ischemia

The role of focal nerve ischemia in this model of peripheral nerve injury has been investigated. Nerve blood flow has been shown to be significantly decreased in the ligated segment during the development of thermal hyperalgesia (Myers et al, 1993). This suggested that the changes in blood flow caused by the ligature compression contributed to the fibre injury. In addition, this study investigated different injuries to the sciatic nerve. The sciatic nerve had either been crushed, made ischemic by ligating the femoral artery or had the surrounding epineurium removed. The pathological changes in the ligature model were less severe than the crush model but greater than those from the femoral artery ligation and epineurial devascularisation. This order also correlated with the average behavioural scores obtained in response to applying heat to the ipsilateral paw. Removal of the

epineurium did not result in a significant hyperalgesia to thermal stimuli. Nerve ischemia has been found sufficient to initiate the development of behavioural hyperalgesia in the ipsilateral paw, when Wallerian degeneration has been produced. It was suggested by Myers & co-workers that the pathological events iniated by nerve ischemia may be more directly related to the development of hyperalgesia than the initial ischaemic insult.

2.3.1.(d) Endoneurial fluid pressure

Bennett and Xie (1988) suggested that partial occlusion of the nerve's vasculature resulted in an intraneural edema the force of which opposes the unyielding ligatures and results in strangulation of the nerve. Endoneurial edema has been a prominent histological finding that presumably results from increased endoneurial fluid pressure (Powell et al, 1989). Wallerian degeneration has been shown to evoke endoneurial plasma extravasation (Powell et al, 1979). It has been suggested that increased endoneurial fluid pressure may be reflected back to the dorsal root ganglia, providing a mechanical stimulus for evoking activity in sensory neurones (Howe et al, 1977; Myers et al, 1988).

Overview

These studies suggest that peri-axonal inflammation, endoneurial fluid pressure and restriction of the perineural blood supply may contribute to the development of the behavioural changes associated with the Bennett & Xie model. Comparable behavioural changes have been obtained using the Seltzer and Kim & Chung models which both use silk to ligate the nerve. This argues against the importance of the chemicals in chromic gut and a marked inflammatory response as

silk causes only a small inflammatory response. Thus, the factors involved in the development of the behavioural changes of the Seltzer and Kim & Chung model also requires investigation. It has been suggested that fibre loss produced by the Bennett & Xie model may result in the behavioural changes associated with this model. This will be discussed in more detail.

2.3.2. ALTERATIONS IN NERVE FIBRES AFTER PERIPHERAL NERVE INJURY

In the model of Bennett & Xie alteration of the fibre spectrum in the nerve distal to the constriction injury has been found to be variable with most observers reporting animal-to-animal variability (Gautron et al, 1990; Basbaum et al, 1991; Carlton et al, 1991; Munger et al, 1992; Nuytten et al, 1992; Coggeshall et al, 1993; Guilbaud et al, 1993). The variability in fibre damage may arise due to several factors. Variability in the tightness of the constriction may be important as chromic gut is not a malleable material and is thus difficult to knot (Nuytten et al, 1992; Ro & Jacobs 1993). Alternatively, the alteration in fibre loss may vary for different strains of rat (Nuytten et al, 1992). Another possibility is that differences in the type of suture material may account for the variability as Maves and co-workers suggested that the chemicals released from chromic gut may be important (Maves et al, 1992; 1993).

2.3.2.(a) Large diameter myelinated fibres

The primary morphological effect of this model to the nerve distal to the ligature is a major disruption in the population of large to medium sized myelinated axons. The proximal nerve has been found to have a relatively normal complement of myelinated fibres (Basbaum et al, 1991; Munger et al, 1992; Coggeshall et al, 1993) although some studies have reported a small loss of large myelinated fibres (Carlton et

al, 1991; Guilbaud et al, 1993). At light magnification level, distal to the injury each nerve had fewer myelinated fibres, much degenerating myelin and Schwann cell process proliferation although many activated Schwann cells appeared normal (Carlton et al, 1991). An acute axonopathy which greatly affected myelinated fibres has been found distal to the ligature (Bennett & Xie, 1988; Gautron et al, 1990; Carlton et al, 1991; Munger et al 1992; Guilbaud et al, 1993). The decrease in large myelinated fibres has been described as almost total although considerable variability in this decrease has been reported (Basbaum et al, 1991; Munger et al, 1992; Nuytten et al, 1992). The loss of myelinated fibres has been reported to range from a minimum of 84% to a maximum decrease of 99% (Carlton et al, 1991). Most studies focused on one time point however Coggeshall and co-workers assessed the fibre size and number over 56 days. Large fibre loss was found by day 3 postoperatively (Coggeshall et al, 1993) since there is evidence that it takes at least 2 days after a peripheral axon is separated from its cell body for morphologic signs of death to become apparent (Maynard et al, 1977). From 3 days until 2 weeks a steady and extensive decline in the numbers of all sizes of fibres occurred with the larger the axon diameter the greater the loss. The decrease in large myelinated fibres was found to be maximal at 2 weeks with recovery reported to begin from between 14 and 28 days which was confirmed by Guilbaud et al, (1993). By 56 days postoperatively the number of large fibres had increased to almost normal values (Coggeshall et al, 1993). However, Guilbaud and co-workers found that a total recovery of large fibres was not complete until week 15 (Guilbaud et al, 1993).

2.3.2.(b) Small diameter myelinated fibres

The loss of small myelinated fibres is not as great as that reported for large myelinated fibres. Decreases (Basbaum et al, 1991; Munger et al, 1992; Nuytten et al, 1992), increases (Coggeshall et al, 1993; Guilbaud et al, 1993) and no change in the

number of small myelinated fibres have been reported (Gautron et al, 1990). The increases are likely the result of regeneration and remyelination (Coggeshall et al, 1993; Guilbaud et al, 1993). In support of remyelination, abnormal but intact thin myelinated sheaths have been found with very short internodal lengths (Basbaum et al, 1991; Coggeshall et al, 1993).

Significant variation in the severity of the loss of the smallest diameter myelinated fibres has been reported (Basbaum et al, 1991). In some fascicles there were no surviving myelinated axons distal to the ligature whereas in others there appeared to be a normal complement of small myelinated fibres. In contrast, a consistent group of small myelinated nerve fibres with a thin myelin sheath has been observed and these were surrounded by endoneurial capillaries in the centre of each nerve fascicle (Nuytten et al, 1992).

2.3.2.(c) Unmyelinated axons

The decrease in the unmyelinated axon population is less extensive but more variable than that obtained for myelinated axons after peripheral nerve injury (Basbaum et al, 1991; Carlton et al, 1991; Munger et al, 1992; Coggeshall et al, 1993). Although the unmyelinated fibres have been found to decrease by approximately 70% by 3 studies other studies have found greater variability (Basbaum et al, 1991; Coggeshall et al, 1993; Carlton et al, 1991). This variability in the results is evident in the percentage decreases obtained in a study involving just 2 animals which reported a decrease in unmyelinated afferents of 34% and 71% (Basbaum et al, 1991). A maximum decrease of 84% was obtained in a study reporting decreases of 62-84% (Carlton et al, 1991).

The typical tightly packed clusters of unmyelinated axons as seen in the proximal segment of the ligatured nerve and in normal nerve have not been found distally (Nuytten et al, 1992; Basbaum et al, 1991). In addition, the morphology of

these axons has been found to change. They were not round and smooth as in the proximal nerve but instead had a crelated appearance. Variability in the size of the unmyelinated axons has been reported (Nuytten et al, 1992). Although a minority of unmyelinated fibres had diameters within the normal range, the majority had pathologically small diameters (Nuytten et al, 1992).

Abnormal unmyelinated fibre bundles have been found by day 5 with maximal changes occurring at day 14 and a return to control conditions by day 28 (Coggeshall et al, 1993). Empty Schwann cell bands and an increase in the number of Schwann cell processes have provided indirect evidence of degeneration of unmyelinated fibres (Dyck & Hopskins, 1972; Ochoa, 1978). Regenerating axons are likely to include unmyelinated axons as well as regenerating myelinated axons before the onset of myelination.

2.3.2.(d) Relationship between fibre loss and pain behaviours

It has been suggested that the development of mechanical hyperalgesia and allodynia is a result of the fibre losses produced by nerve ligatures. For example there may be a release of inhibition of nociceptive primary afferent input into the spinal cord dorsal horn as a result of the elimination of large myelinated axons (Gautron et al, 1990; Garrison et al, 1991; Laird & Bennett ,1992; Guilbaud et al, 1993). The initiation of pain-related behaviours has not been closely correlated with the development of large myelinated fibres degeneration as most studies have focused on the changes when the behavioural changes are near maximal (Gautron et al, 1990; Munger et al, 1992; Carlton et al, 1991). Coggeshall and co-workers found that the initiation of pain coincided with the initial degeneration and demeylination reported from day 3 onwards. Twenty eight days postoperatively, most of the large myelinated fibres had been lost and the signs of hyperalgesia had almost resolved. Therefore no relation between the disappearance of the pain related behaviour and the large fibre

population was established (Coggeshall et al, 1993). Two additional studies have also found that the behavioural symptoms disappeared although the large myelinated population was depleted (Gautron et al, 1990; Guilbaud et al, 1993).

In addition, it has been suggested that the small myelinated and unmyelinated axons which are spared injury may be responsible for the pain related behaviour (Gautron et al, 1990; Basbaum et al, 1991). The pathological state of those which remain may contribute to the behavioural changes as the properties of these nociceptive afferents may change (Basbaum et al, 1991; Munger et al, 1992). Carlton and co-workers hypothesised that the input from surviving axons in the distal segments results in increased activity in the neuroma which in turn sends signals centrally that are interpreted as nociceptive by the animal.

The simultaneous presence of both degenerating and regenerating fibres of relatively thin diameter has been suggested to be the major cause of the pain related behaviours (Guilbaud et al, 1993). It has been suggested that the unequal rates of successful regeneration and or remyelination may underlie the difference between painless diabetic neuropathy in which the success of regeneration and remyelination is minimal and sensation is decreased, and painful neuropathies in which there is a greater incidence of successful reinnervation (Britland et al, 1990).

2.4. NEUROPEPTIDE CHANGES IN DORSAL ROOT GANGLIA AND SPINAL CORD AFTER NERVE INJURY

2.4.1. COMPLETE NERVE TRANSECTION

Transection of a peripheral sensory nerve leads to complex metabolic changes in affected sensory ganglion nerve cell bodies and their terminals in the dorsal horn of the spinal cord. The expression of neuropeptides in primary sensory neurones is thus plastic with decreases or dramatic increases (including de novo synthesis) occurring. Depending on the rate at which the neuropeptide is transported and released, this will produce alteration in the levels found in cell bodies and their terminals. Radioimmunoassay, immunohistochemical and in situ hybridisation experiments have been carried out to determine the neuropeptide changes in the spinal cord and dorsal root ganglion (reviewed by Hokfelt et al, 1994). Neuropeptide changes can be grouped into (i) those that decrease: substance P (SP), somatostatin, calcitonin-gene related peptide (CGRP) and (ii) those that increase: vasoactive intestinal peptide (VIP), galanin and NPY (McGregor et al, 1984; Shehab & Atkinson, 1986; Villar et al, 1989; Wakisaka et al, 1991b; Knyihar-Csillik et al, 1993). The majority of this work has been carried out in the rat with limited studies in other species.

The first evidence for plasticity in neuropeptide expression following peripheral axotomy was presented in 1979 (Jessell et al, 1979). Sciatic nerve section produced a 75-80% depletion of ir-SP in the ipsilateral dorsal horn but did not change immunoreactivity levels in the ventral horn. This resulted in an area which was depleted of ir-SP involving the medial 2/3rds of lamina I-II of lumbar segments L4 & L5 (Barbut et al, 1981). A decrease in immunoreactivity may indicate increased release or decreased synthesis of the peptide and hence in situ hybridisation experiments have been performed to determine the levels of mRNA before and after

transection since the latter gives information on synthesis. Preprotachykinin (PPT) mRNA which codes for SP has been found to be reduced in small diameter dorsal root ganglion cells following sciatic nerve transection (Nielsch et al, 1987; Nielsch & Keen, 1989; Noguchi et al, 1989). This reduction in mRNA has been shown to be less marked following peripheral crush when compared with transection. In contrast, *de novo* synthesis of PPT mRNA has been demonstrated in medium & large dorsal root ganglion neurones in response to nerve injury (Noguchi et al, 1994).

Somatostatin has been shown to decrease in the dorsal root ganglia and their terminals in the spinal cord following peripheral axotomy but was unchanged following sciatic nerve crush (McGregor et al, 1984; Shehab & Atkinson, 1986). A reduction in ir-somatostatin has been reported in lamina II of the monkey spinal cord after transection of the sciatic nerve (Zhang et al, 1993a). Immunoreactive-CGRP levels have been reported to decrease in L5 dorsal root ganglia after sciatic nerve transection reaching minimal levels during the second post operative week (Dumoulin et al, 1991; Doughty et al, 1991). Somatostatin and CGRP mRNA have been found to be reduced in rat dorsal root ganglia ipsilateral to the sciatic nerve transection (Noguchi et al, 1993). There are two types of mRNA for CGRP (α & β) which have been found to be differentially distributed in sensory neurones. Large dorsal root ganglion cells have been found to contain the α form of mRNA and small cells contain both types (Noguchi et al, 1990). The levels of both have been reported to decrease in dorsal root ganglia, seven days after sciatic nerve transection in the rat (Noguchi et al, 1990). In contrast, an increase in ir-CGRP in spinal motoneurones occurs at this time (Noguchi et al, 1990). In the monkey a reduction in the number of ir-CGRP and ir-SP dorsal root ganglia neurones has been reported as has a decrease in the mRNA levels of these two peptides (Zhang et al, 1993a).

Immunoreactive-cholecystokinin (CCK) in the ipsilateral lumbar dorsal horn has been reported to not change or decrease after sciatic nerve transection (McGregor et al, 1984; Shehab & Atkinson, 1986). The identity of this immunoreactivity is

uncertain. Evidence has been presented that CCK like immunoreactivity may represent cross reactivity with CGRP or a CGRP-like peptide or an unknown CCK like peptide (Ju et al, 1986; Hokfelt et al, 1988). mRNA for CCK has been found to increase in dorsal root ganglion ipsilateral to the nerve transection which may suggest increased release since the immunoreactivity levels seem to decrease (Verge et al, 1993; Xu et al, 1993a).

Immunoreactive-VIP has been found to increase in the rat dorsal root ganglia and spinal cord following peripheral nerve transection (McGregor et al, 1984; Shehab & Atkinson, 1986; Shehab et al, 1986). In the spinal cord this increase has been demonstrated by immunocytochemistry to be confined to the substantia gelatinosa of the ipsilateral cord (McGregor et al, 1984). Similar increases to VIP have been observed for a related peptide with N-terminal histidine and C-terminal isoleucine amide (PHI) (McGregor et al, 1984; Hokfelt et al, 1987; Villar et al, 1989). In the dorsal root ganglia the increase in ir-VIP has been found to occur in mainly small and also some medium diameter dorsal root ganglion cells (Shehab et al, 1986; Zhang et al, 1995b). Small neurones containing ir-VIP have also been found to contain ir-galanin (Kashiba et al, 1992). Four days after transection, half of the dorsal root ganglia immunoreactive for VIP were also immunoreactive for CGRP (Doughty et al, 1991). The increase in immunoreactivity in the cell bodies may be due to an accumulation as a result of decreased axonal transport or alternatively due to an upregulation of synthesis. To investigate this *in situ* hybridisation experiments have been employed. The change in immunoreactivity was subsequently shown to be due to a dramatic induction of VIP mRNA from an undetectable level of expression in normal rats (Noguchi et al, 1993).

In the rat the changes in pituitary adenylate cyclase activating peptide (PACAP) after sciatic nerve transection have been recently investigated (Zhang.Q. et al, 1995). The N-terminal sequence of this 38 amino acid peptide has been shown to be 68% homologous to VIP (Miyata et al, 1989). This study by Zhang & co-workers

found a moderate increase in ir-PACAP levels in the ipsilateral superficial dorsal horn by 3 days at which time more than 50% of all dorsal root ganglion neurones expressed ir-PACAP. This increase was found to occur mainly in the medium and large sized neurones.

Galanin immunoreactivity has been demonstrated in small medium and large dorsal root ganglion neurones (Villar et al, 1989; Kashiba et al, 1992). An increase in ir-galanin has been found in the midportion of the superficial dorsal horn ipsilateral to the transected nerve (Villar et al, 1989). This corresponded to the area where ir-subP has been shown to decrease and ir-VIP increase. The number of dorsal root ganglion neurones immunoreactive for galanin was greater than those immunoreactive for VIP at L5 spinal level (Kashiba et al, 1992). Galanin and VIP have often been found to be co-localised in neurones with decreased levels of ir-SP and ir-CGRP (Xu et al, 1990; Doughty et al, 1991; Kashiba et al, 1992). Transection was also found to cause an increase in ir-galanin in the dorsal root ganglia of the monkey, cat and guinea-pig (Arvidsson et al, 1991b; Zhang et al, 1993a, Rydh-Rinder et al, 1996). Induction of galanin mRNA has been demonstrated in rat dorsal root ganglia following transection (Noguchi et al, 1993).

A subpopulation of dorsal root ganglion neurones have been found to show NPY immunoreactivity (Wakisaka et al, 1991b, Kashiba et al, 1994). Immunohistochemical changes occurred mainly in large and some medium sized dorsal root ganglion neurones (Wakisaka et al, 1992; Zhang et al, 1993b; Kashiba et al, 1994). Immunoreactive-NPY has been reported to be present in 20-30% of ipsilateral dorsal root ganglion neurones in lumbar regions L4-5 (Kashiba et al, 1994). In this study immunohistochemical analysis combined with retrograde tracing detected ir-NPY in cutaneous and muscular sensory neurones but not in visceral neurones. In large cells, the newly synthesised NPY has been found to be co-localised with galanin (Kashiba et al, 1994). In the dorsal horn of the guinea-pig a decrease in ir-NPY in lamina I & II and an increase in laminae III has been found (Rydh-Rinder et al, 1996).

In contrast to the rat, changes in NPY have been reported mainly in the small dorsal root ganglion neurones of the guinea-pig. The alteration of NPY following peripheral nerve cut using RNA blot and *in situ* hybridisation has suggested that the NPY detected is the result of *de novo* translation of NPY mRNA (Noguchi et al, 1993). The neurones exhibiting NPY mRNA induction are a subpopulation of the largest sized labelled neurones with only a few small neurones labelled.

The presence of NPY immunoreactivity has been found to increase in the spinal cord after peripheral nerve transection (Wakisaka et al, 1991b; Kashiba et al, 1994; Zhang et al, 1995a & b). This is contrary to an earlier result reported by Gibson and co-workers which found no change in ir-NPY in the rat lumbar spinal cord almost 3 weeks following sciatic nerve transection (Gibson et al, 1984). Two weeks after injury, immunoreactive NPY has been found to increase in laminae III-V of the spinal cord (Wakisaka et al, 1991b) and III-IV (Zhang et al, 1995b). This increase occurred in axons and varicosities in the ipsilateral spinal cord only (Wakisaka et al, 1991b; Zhang et al, 1993). Ohara and co-workers found that changes in ir-NPY in the dorsal horn were more intense and sustained in response to transection compared to crushing the nerve with forceps (Ohara et al, 1994). They also found that lumbar dorsal rhizotomy performed at the same time as sciatic nerve transection prevented the development of NPY immunoreactivity in laminae III & IV suggesting that the NPY was contained within large primary afferent fibres.

Neuropeptide changes are not unique to sciatic nerve section. They have also been found to occur when the mandibular branch of the trigeminal nerve has been cut (Atkinson & Shehab, 1986). Immunoreactive-VIP has been found to increase whereas SP, cholecystokinin and somatostatin immunoreactivity decrease in the trigeminal ganglia. Four weeks after pelvic nerve transection there was a decrease in ir-VIP and no change in ir-galanin or ir-SP in the dorsal sacral spinal cord of the cat. Transection of the mandibular nerve resulted in a marked increased galanin, VIP and NPY mRNA. In contrast, transection of the vagal nerve resulted in a marked increase

in galanin and VIP in the nodose ganglia with only a moderate increase in NPY mRNA (Zhang et al, 1996). This change in mRNA has been found in small neurones of the nodose ganglia whereas the NPY increase has been found in medium and large sized trigeminal ganglia (Wakisaka et al, 1993; Zhang et al, 1996). In contrast, there was an increase in ir-VIP and galanin and a decrease in ir-SP in the dorsal sacral cord after transection of the pudendal nerve (Anand et al, 1991).

2.4.2. PARTIAL NERVE INJURY

Alterations in neuropeptides also occur when a nerve is partially injured (Wakisakaka et al, 1992, Nahin et al, 1994) such as in the mononeuropathy model of Bennett and Xie (1988). Immunoreactive-SP (Cameron et al, 1991; Munglani et al, 1995) and ir-CGRP (Cameron et al, 1991) have been reported to decrease in the dorsal root ganglia and superficial dorsal horn ipsilateral to the peripheral neuropathy. Similar to transection, an increase in ir-NPY has been found in medium and large dorsal root ganglion neurones (Wakisaka et al, 1992b). Galanin, VIP and NPY mRNA all increased from non-detectable levels from day 3 with maximum expression by day 14 in the dorsal root ganglia ipsilateral to peripheral nerve injury. A maximum decrease in the messenger RNA levels for SP and CGRP occurred during the second postoperative week (Nahin et al, 1994).

An increase in ir-NPY in laminae III-V axons and varicosities was found in the spinal cord at 14 days after partial nerve injury (Wakisaka et al, 1992). An increase in ir-NPY has also been reported 28 days after partial nerve injury with no significant changes in laminae I-II and an increase in laminae III-IV (Munglani et al, 1995).

2.5. THE ROLE OF THE SYMPATHETIC NERVOUS SYSTEM

Historically, the observation that patients with nerve injury had cold skin led to the idea of blocking sympathetic activity as it was suggested that the cold skin was related to sympathetic hyperactivity which excited nociceptive fibres within the symptomatic area (Leriche, 1939). Although, the role of the sympathetic nervous system in mediating abnormal behaviours associated with peripheral nerve injuries remains unclear, sympathectomy has been continued to be used to treat such patients. Several possibilities to explain the role of the sympathetic system have been suggested and will be discussed below. The simplest explanation for the beneficial effects of sympathectomy in neuropathic pain is that some form of link exists between the autonomic system and primary afferent fibres. A normal physiological coupling may exist between the sympathetic system and primary afferents whereby chemical changes in the microenvironment result in the sensitisation of primary afferent terminals. Alternatively, there may be pathological coupling either at the site of nerve damage or more peripherally or centrally. Finally some evidence suggests that abnormal central processing may be involved.

2.5.1. SYMPATHETIC EFFERENTS AND AUTONOMIC EFFECTOR ORGANS

It has been suggested that sympathetic efferents can influence primary afferents through autonomic effector organs. The clinical observations of temperature differences and of sweating in the hands or feet of neuropathic patients (those with RSD, causalgia or SMP) has lead to the proposal that sympathetic functions are disturbed in these patients. Disturbance of the vasoconstrictor system may be

involved leading to changes in the regulation of blood flow in the distal extremities (Blumberg, 1993).

Only some patients respond well to sympathectomy (DeTakats, 1943; Bonelli et al, 1983; Driessen et al, 1983; Wang et al, 1985; Arner, 1991; Blumberg & Janig 1993; Blumberg et al, 1994). In clinical case studies of patients who responded positively to sympatholytic treatment, an increase of hydrostatic pressure by venous congestion has been found to enhance the pain whilst an occlusion of arterial supply has relieved the painful symptoms before the ischaemic conduction block of afferent fibres could have occurred (Gracely et al, 1990; Blumberg, 1992). Interrupting the circulation of the distal part of the affected extremity has resulted in complete relief of pain for the duration of the interruption (DeTakats, 1943; Gracely et al, 1990). Thus it has been suggested that changes of the microcirculation might be involved in the generation of pain. Blumberg has hypothesised that an indirect sympathetic-sensory coupling occurs via the vascular system. According to this hypothesis the initial nerve injury produces a massive input to the spinal cord. Some spinal circuits are sensitised by nociceptive afferent input which leads to an abnormal discharge pattern in sympathetic vasoconstrictor neurones. This results in an increase in vasoconstriction on the postcapillary side compared with the precapillary side, inducing increased capillary filtration pressure. The related increase in interstitial pressure activates nociceptive afferent fibres and hence the pain is essentially akin to deep muscle pain. Supporting this, animal models have demonstrated that peripheral nerve damage can result in abnormal reflex behaviour of sympathetic vasoconstrictor nerves in skin (Blumberg & Janig, 1985; Janig & Koltzenburg, 1991). However, direct evidence is missing as clinical observations and animal experiments have provided no evidence to support the assumption that the activity of sympathetic neurones (sympathetic tone) is increased (Blumberg, 1991; Janig & Koltzenburg, 1991; reviewed by Blumberg 1992).

2.5.2. PHYSIOLOGICAL COUPLING BETWEEN SYMPATHETIC EFFERENTS AND PRIMARY AFFERENTS

It has been suggested that the sympathetic system might act indirectly on primary afferents through precipitating a cascade of chemical changes in the microenvironment surrounding the axon terminals leading to excitation or sensitisation of afferent endings (Janig & Koltzenburg, 1991; Koltzenburg & McMahon, 1991; Campbell et al, 1992; Janig & Koltzenburg, 1992; Gracely et al, 1992).

Bradykinin has been found to produce mechanical hyperalgesia in peripheral tissues (Beck et al, 1974; Chahl & Iggo, 1977). In the experiments of Levine et al (1986b) bradykinin hyperalgesia was present in normal skin whereas noradrenaline induced hyperalgesia was only observed in chloroform treated (chemically inflamed) skin. Repetitive topical application of chloroform induced hyperalgesia which was enhanced by noradrenaline. Levine and co-workers suggested that noradrenaline released from sympathetic endings activated other sympathetic endings through an alpha-2 adrenoceptor mechanism resulting in the liberation of prostaglandins. The latter would then sensitise nociceptors (Levine et al, 1986a). Both bradykinin- and noradrenaline-induced hyperalgesia were found to be dependent on the presence of an intact sympathetic innervation and the production of prostaglandins as they were blocked both by sympathectomy (Levine et al, 1986b) and by previously administered non-steriodal anti-inflammatory agents (Lembeck et al, 1976; Levine et al, 1986b). The latency to onset of hyperalgesia produced by PGE_2 has been found to be significantly shorter than the latency to onset of bradykinin-induced hyperalgesia suggesting an indirect mechanism for the latter (Taiwo et al, 1987). Taiwo & Levine (1988) suggested that bradykinin- and noradrenaline-induced hyperalgesia (in chloroform treated skin) share a common mechanism as they have similar onset latencies (Taiwo & Levine 1988). The hyperalgesic effects of PGE_2 and PGI_2 have

been distinguished by the duration of the hyperalgesia they produced (Taiwo et al, 1987) and were thus used by Taiwo & Levine (1988) to compare the duration of bradykinin- and noradrenaline-induced hyperalgesia. They found that bradykinin and PGE_2 induced hyperalgesia had a similar duration as did noradrenaline and PGI_2 induced hyperalgesia. In addition, the prostaglandin antagonist, SC19220, selectively inhibited PGE_2 induced hyperalgesia but antagonised the effects of bradykinin suggesting that bradykinin induced hyperalgesia is mediated by PGE_2 and noradrenaline induced hyperalgesia by PGI_2. These proposals of Levine and co-workers have been challenged by the experiments of Koltzenburg et al (1992) who found that the sensitisation of nociceptors by bradykinin does not depend on sympathetic neurones.

2.5.3. PATHOLOGICAL COUPLING BETWEEN SYMPATHETIC EFFERENTS AND PRIMARY AFFERENTS

Early ideas favoured ephatic electrical cross talk as a means of communication between incoming and outgoing fibres. Since the sympathetic system is tonically active it was suggested that sympathetic efferents and primary afferent fibres may be involved in cross talk. It was proposed that artificial synapses formed at the site of the lesion on the peripheral nerve (Doupe et al, 1944). Thus, impulses leaving the spinal cord in sympathetic fibres were transmitted at the artificial synapse by ephatic transmission to small somatic afferents fibres resulting in pain. According to this hypothesis a guanethidine block would have no effect since transmission is electrical. The introduction of Hannington-Kiffs method (1974) of blocking the sympathetic nerve endings however has not supported this proposal. Guanethidine blocks have been found to be as successful in relieving the pain as are blocks of sympathetic chain and ganglia (Hannington-Kiff 1974; Schott 1986; Bonica, 1990).

Clinically, the activation of sympathetic post-ganglionic neurones or application of noradrenaline to painful skin has been found to exacerbate pain & hyperalgesia in patients with causalgia (Wallin et al, 1976 ; Wiesenfeld-Hallin & Hallin 1984; Torebjork et al, 1995) while sympathetic interventions can abolish hyperalgesia and pain (Hannington-Kiff, 1974; Schott, 1986; Bonica, 1990). Therefore it has been suggested that the neuropathic pain syndrome of certain patients is maintained by the release of noradrenaline from postganglionic sympathetic fibres activating primary afferent fibers via alpha adrenoceptors (Campbell et al, 1992). Such an hypothesis requires the expression of adrenoceptors by primary afferents. Evidence for this has come from the experimental analysis of the action of catecholamines on damaged peripheral nerve. It was demonstrated that sprouting fibres trapped in a neuroma formed by regenerating axons after transection became sensitive to the alpha receptor mediated action of adrenaline (Wall & Gutnick, 1974 a & b). Exposure to adrenaline and noradrenaline induced and increased impulse discharge which was stopped by the non-selective alpha antagonist, phentolamine, and unaffected by isoprenaline (a beta agonist). Later studies confirmed that fibres in the neuroma (Devor & Janig, 1981; Korenmann & Devor, 1981; Scadding, 1981; Blumberg & Janig, 1984; Burchiel, 1984b; Habler et al, 1987; Welk et al, 1990) as well as non-axotomised unmyelinated C fibres with receptive terminals in the territory of the partially lesioned nerve (Habler et al, 1987; Sato & Perl 1991; Budewiser et al, 1993; Selig et al, 1993) responded to catecholamines. In addition, it was found that sufficient noradrenaline was released by stimulation of the postganglionic sympathetic fibres to evoke firing (Devor & Janig, 1981; Blumberg & Janig, 1984; Habler et al, 1987) by activating alpha adrenoceptors. Devor & Janig (1981) found that repetitive stimulation of the lumbar sympathetic trunk resulted in afferent impulses in primary afferents and this was blocked by the alpha adrenergic antagonist phentolamine whereas the beta adrenergic antagonist propranolol had no effect.

Ectopic activity generated by the cell bodies of damaged afferents may also be influenced by activity in sympathetic postganglionic fibres (Devor et al, 1991). After sciatic nerve transection, noradrenergic perivascular axons were found to sprout into the dorsal root ganglia forming basket-like structures around large diameter axotomised sensory neurones. Nerve injury thus resulted in a new site of coupling between the sympathetic and primary afferent system. It has been suggested that this may be a further origin for abnormal discharge following peripheral nerve damage (McLachlan et al, 1993).

The observations of Wall & Gutnick first raised the possibility that the damaged fibres expressed adrenoceptors. A significant elevation of clonidine binding sites in the neuroma and in the superficial dorsal horn (where many unmyelinated afferents terminate) has been found by McMahon (1991). In addition, Williams and co-workers (1991) have suggested that the synthesis of this receptor may be increased after peripheral nerve injury.

Radioligand binding studies in a variety of mammalian species and tissues have indicated that alpha 2 adrenoceptors exist in at least 3 pharmacological distinguishable types 2a, 2b and 2c (Ruffolo et al; 1987). The rat spinal cord contains alpha 2a and 2c adrenoceptor binding sites whereas alpha 2b adrenoceptors were not detectable (Uhlen & Wikberg, 1991; Uhlen et al, 1992). Approximately 96% of the alpha 2 adrenoceptors in the spinal cord have been shown to be of the alpha 2a type whereas about 4% were of the alpha 2c type (Uhlen et al, 1992). However, the roles of these receptor subtypes in the modulation of nociceptive information are unclear.

2.5.4. THE ROLE OF THE SYMPATHETIC NERVOUS SYSTEM IN PARTIAL NERVE INJURY MODELS

If the animal models of nerve damage are relevant to the human clinical syndromes then a modification of the pain states by altering sympathetic activity should occur.

2.5.4.(a) The Bennett & Xie model

With this model there is evidence that the sympathetic nervous system has a role in the development and maintenance of some abnormal pain-related behaviours. The development of behavioural sensitisation to mechanical, heat and cold stimuli in the partially denervated area of the hindpaw in the rat has been investigated. Two groups have assessed the effects of the sympatholytic agent guanethidine administered either subcutaneously for 4 consecutive days before surgery or 10 days after surgery (Neil et al, 1991) or intravenously for seven days starting one week after surgery (Perrot et al, 1993). The effect of a surgical sympathectomy on pain related behaviours has been investigated more recently (Desmeules et al, 1995). Sympathectomy significantly reduced the abnormal response to heat measured by determining the withdrawal threshold to heat by submersing the limb in a series of controlled ascending temperature water baths (Neil et al, 1991) or by determining the struggle latency to paw immersion in a 45°C water bath (Desmeules et al, 1995). The hindpaw was submersed into a water bath at 10°C to determine the latency to withdrawal or struggle response to a cold stimulus (Neil et al, 1991; Perrot et al, 1993; Desmeules et al, 1995). The abnormal reactions to a cold stimulus in neuropathic rats was almost completely suppressed (Neil et al, 1991; Perrot et al, 1993). In contrast, the abnormal response to mechanical pressure measured by the Randall-Selitto analygesymeter was found to be unchanged in 2 of these studies (Neil et al, 1991; Desmeules et al, 1995). This differs from the study by Perrot and co-

workers (1993) which found that sympathectomy performed by intraperitoneal injection of guanethidine resulted in a reduction in the mechanical threshold elicited by hind-paw pressure.

Wakisaka and co-workers (1991a) compared the temperature differences of the plantar skin of the hind paws of normal rats and those with a loose ligation on one side and a sham operation on the opposite side. In normal rats little temperature difference between the two normal hind paws was obtained. In contrast, the temperature difference obtained by subtracting the sham-operated plantar hind paw temperature from the operated hindpaw temperature resulted in abnormally large temperature differences. The skin on the nerve injured side was warmer than normal during the first 10 days postoperatively and colder than normal at 3-4 weeks postoperatively. Sympathetic block delivered at 2 or more weeks post injury was found to be without effect (Wakisaka et al, 1991). Until recently, the skin temperature changes after painful neuropathies have been thought to reflect abnormal vasoconstrictor reflexes with cold skin being associated with inappropriately high levels of activity in the sympathetic cutaneous vasoconstrictor neurones and a low level of activity with warm skin. A relationship between skin temperature and activity in sympathetic neurones has not been proven. This is probably a misconception which is supported by the findings of this study by Wakisaka's group.

2.5.4.(b) Kim & Chung model

Surgical sympathectomy performed by removing the sympathetic chain bilaterally from the L2 to L6 level before nerve injury produced similar results to performing the sympathectomy after surgery in the Kim & Chung model (Kim et al, 1993; Kinnman & Levine, 1995). At 1 week prior to and 1, 3 and 5 weeks after nerve injury sympathectomy alleviated both mechanical allodynia and heat hyperalgesia (Kim et al, 1993). Sympathectomy has been found to alleviate the mechanical

sensitivity within 30 minutes and the heat hyperalgesia within one day. Thus it has been suggested that the sympathetic nervous system contributes to both the initiation and the maintenance of nerve injury-induced increased mechanical sensitivity. Sham sympathectomy had no effect on either mechanical allodynia or thermal hyperalgesia suggesting that the sham operation per se did not affect the abnormal pain behaviours (Kim & Chung, 1991). Sympathetic postganglionic neurones at the level of spinal injury can contribute to neuropathy symptoms independent of input from preganglionic neurones as a lesion of preganglionic fibres to the L5 level had no significant effect on sensory abnormalities (Kinnman & Levine, 1995).

2.5.4.(c) Seltzer Model

Sympathetic block using guanethidine administered by intra-peritoneal injection was first reported to alleviate behavioural changes in an experimental peripheral neuropathy by Shir & Seltzer (1991). The main observation of this study was that sympathectomy performed several months after partial sciatic nerve injury alleviated the sensory disorders bilaterally produced by non-noxious and noxious stimuli. The presence of sympathetic activity was not essential for the production of touch allodynia as it did not prevent the postoperative appearance of touch-evoked allodynia. This suggests that the coupling between sympathetic efferents and low threshold mechanoreceptive A fibres gradually increases with time. Tracey and co-workers found that subcutaneous injection of noradrenaline exacerbated the mechanical and thermal hyperalgesia in this model while subcutaneous injection of yohimbine, the alpha 2 adrenergic antagonist, diminished the hyperalgesia. In contrast, noradrenaline had no effect in control animals. They found that guanethidine treatment diminished the hyperalgesia associated with this model. From these results they suggested that behavioural changes are maintained by activation of alpha 2 adrenoceptors located on post-ganglionic sympathetic terminals and that these

nociceptors are sensitised by increased synthesis of prostaglandins by activation of these receptors as proposed by Levine (Tracey et al, 1995a).

Experimentally, capsaicin which destroys most C fibres and some A delta fibers in peripheral nerves had no effect on the touch-evoked allodynia and mechanical hyperesthesia produced by partial sciatic nerve injury using the Seltzer model (Shir & Seltzer, 1990). This study thus suggested that these disorders were mediated by myelinated fibers. However, thermal hyperalgesia failed to develop in these capsaicin-treated rats suggesting that the thermal hyperalgesia was mediated by heat-nociceptive C fibers. In agreement, clinical results from differential nerve blocks have suggested that the noradrenaline-induced ongoing pain and heat hyperalgesia were signalled by unmyelinated afferents, while touch-evoked pain and cold hyperalgesia were signalled by myelinated afferents (Torebjork et al, 1995). Nociceptor sensitisation has been suggested to be at least partly responsible for the reduction of the perceptual threshold for heat-evoked pain and hyperalgesia present when the skin has been injured (for review see Meyer et al, 1992).

2.6. PROPERTIES OF PRIMARY AFFERENTS IN THE BENNETT AND XIE MODEL

Animal studies have reported that changes in the properties of neurones on the nerve injured side after peripheral nerve injury may contribute to the abnormal behavioural changes which occur (Paleck et al, 1992a; b; Sotgiu et al, 1992; Laird & Bennett, 1993; Sotgiu, 1995 a & b; Koltzenburg et al, 1994). This can be studied either by investigating the properties of the primary afferents beyond the ligature and before they enter the spinal cord or by directly investigating the properties of spinal neurones (see section 2.7.3.). As cited previously there is extensive degeneration of afferents distal to the ligature and this is probably the reason for the paucity of studies on the properties of fibres distal to the ligature.

However, Koltzenburg and co-workers found a significant increase in the mechanical threshold force of mechano-heat-sensitive unmyelinated primary afferents (C-fibers) in the rat on the injured side compared to normals (Koltzenburg et al, 1994). They found that the mean heat response appeared to be increased after partial nerve injury with more spikes evoked by a standard heat stimulus at 2 weeks postoperatively compared to before injury (Koltenburg et al, 1994). The most outstanding change in the properties of primary afferents proximal to the ligature is the occurrence of ectopic discharge.

2.6.1. ECTOPIC IMPULSES

An increase in spontaneous activity in primary afferent fibres may be responsible for the development of continuous pain and may also contribute to the maintenance of allodynia and hyperalgesia (Xie & Xiao, 1990; Kajander & Bennett, 1992; Devor, 1994; Han et al, 1994). Evidence from both human and animal studies

supports a correlation between spontaneous firing in primary afferents and pain (Nystrom & Hagbarth, 1981; Devor, 1994; Seltzer et al, 1991; Gracely et al, 1992). Spontaneous discharges could be generated in the periphery (from sensory endings) or from the neuroma at the injury site or in the dorsal root ganglia. Individual axons may contain more than one locus of afferent impulse generation. Abnormal activity may also originate from zones of demyelination along the nerve (Kajander & Bennett, 1992; Devor, 1994). Studies on ectopic impulse generation have mostly examined afferent neurones with myelinated axons probably because recordings from these fibres are relatively easy to obtain (Burchiel, 1984 a & b; Devor & Bernstein, 1982; Devor & Janig, 1981; Meyer et al, 1985; Scadding, 1981; Wall & Gutnick, 1974; Welk et al, 1990; Devor, 1994; Janig, 1988). Reports on the incidence of spontaneous discharges after nerve injury have given varying results probably depending on the site of origin, the time since the injury and the type of nerve injury as will be described below.

2.6.1.(a) Ectopic impulses and complete nerve transection

(ai) Generation of impulses in dorsal root ganglion cells

After nerve transection, dorsal root ganglion cells increase their tendency to discharge and to bombard the spinal cord with ectopic sensory signals. Kirk in 1970, first suggested that the dorsal root ganglia became spontaneously active after peripheral nerve transection. This was later substantiated by several studies (Wiesenfeld & Lindblom, 1980; De Santis & Duckworth, 1982; Wall & Devor, 1983; Burchiel 1984 a, b; Russell & Burchell, 1988). It has been found that a small percentage of neurones in normal dorsal root ganglia in anaesthetised rats are spontaneously active but this percentage increases after peripheral transection (Wall & Devor, 1983). The incidence of spontaneous discharge originating from the dorsal

root ganglia has been reported as a maximal increase of 6% over normals (Russell & Burchiel, 1988; Wall & Devor, 1983). Sciatic nerve transection was found to evoke spontaneous impulse generation from the dorsal root ganglion cell bodies of myelinated axons (Aβ & Aδ), but not from the cell bodies of unmyelinated axons (Wall & Devor, 1983). After peripheral axotomy, activity originating in fibres at the injury site is typically fast and regular with intermittent bursts whereas that from the dorsal root ganglia is predominantly a slower, random activity (Wall & Devor, 1983; Burchiel, 1984a). The dorsal root ganglion has also been found to be the source of spontaneous discharges recorded from sensory nerve fasicles central to human amputation neuromas as these discharges persisted after local anaesthetic block of the neuroma (Nystrom & Hagbarth, 1981).

(aii) Generation of impulses at the neuroma

Transection of a peripheral nerve disconnects the spinal cord from peripheral sensory end organs. A neuroma is formed by cut fibres being unable to regenerate to their original target tissue. Wall & Gutnick proposed that outgrowing axon sprouts are a novel and abnormal source of afferent barrage (Wall & Gutnick 1974 a & b). They found that dorsal root filaments terminating in the neuroma consistently showed ongoing activity in the absence of any intentional stimulus. Kirk (1974) suggested that some of this activity must have originated in intact proprioceptive and thermo-sensitive fibres as the active roots also contained afferents from nerves which had not been cut. The ongoing activity in these dorsal root filaments was abolished by perfusing the neuroma with lidocaine (Wall & Gutnick, 1974b). Wall & Devor reported that much of the spontaneous firing that occurs after neuroma formation originates at the site of injury, with the remainder emanating from the dorsal root ganglia (Wall & Devor, 1983). Fibres within the developing neuroma exhibit spontaneous, rhythmic, high frequency (up to 100 Hz) bursting discharge activity and

mechanosensitivity (Burchiel, 1984a). This activity is observed as early as 2-3 days after peripheral nerve transection and can be abolished by either local anaesthesia or removal of the neuroma (Burchiel, 1984a). Pressure and adrenaline applied locally to the neuroma increased the frequency of activity and local lidocaine abolished it.

Govrin-Lippmann & Devor (1978) made recordings from the peripheral nerve distal to the dorsal root ganglion but proximal to the cut end of a transected nerve. This study confirmed that impulses are generated at the site of injury as resection of the nerve 5-10mm proximal to its cut end or application of lignocaine abolished all ongoing activity. The ability of the neuroma to generate ongoing activity after transection may be subject to strain related genetic control. In the study of Hao & Wiesenfeld-Hallin (1994) ongoing activity was recorded in a much higher percentage of nerve filaments in Wistar-Kyoto rats compared to Sprague-Dawley rats.

2.6.1.(b) Ectopic impulses and partial nerve injury

(bi) Generation of impulses in dorsal root ganglion cells

There is evidence that much of the spontaneous firing occurring in primary afferents during the early response to nerve injury originates in the dorsal root ganglia (Kajander et al, 1992; Xie et al, 1995). Thirty percent of injured A beta & 15% of A delta axons exhibited spontaneous discharge which originated in the dorsal root ganglia at one & three days postoperatively (Kajander & Bennett 1992; Kajander et al, 1992). Kajander et al, (1992) studied the effect of acute transections at various points along the injured sciatic nerve. Transections just proximal to the nerve injury and just distal to the dorsal root ganglia failed to prevent the discharge, but spontaneous discharge disappeared when the transection was made just proximal to the dorsal root ganglia.

To determine whether spontaneous action potentials originate from the dorsal root ganglion cell body, isolated dorsal root ganglion neurones were studied using the whole cell patch technique (Study & Kral 1996). Spontaneous firing was found in 20% of neurones from ganglia on the side with nerve injury and only 3% of neurones from control ganglia. Neurones were characterised by size roughly corresponding to C (small), A delta (medium) and A beta (large) fibres. The greatest incidence of spontaneous activity was apparent in medium sized neurones although all three groups showed activity. Two types of spontaneous activity have been reported in the dorsal root ganglion cells with some cells showing both types of activity (Study & Kral, 1996). Random action potentials or short bursts of 2-4 rapid spikes with an average frequency of 0.5-3Hz have been shown to be more common than long trains of rapid, regular action potentials at 5-25Hz with occasional random single action potentials (Study & Kral, 1996). It was suggested that the pattern of activity may be dependent on the degree of depolarisation rather than being specific for a particular cell type (Study & Kral, 1996). Spontaneous resting potential fluctuations occurred in both control and nerve injured neurones and triggered the spontaneous, random action potentials in neurones in nerve injured rats (Study & Kral, 1996). A correlation was found between the presence of spontaneous firing after nerve injury and the action potential threshold which was substantially more negative in spontaneously active neurones compared with controls (Study & Kral, 1996). These results suggested that the dorsal root ganglion cell body was the source of this activity.

In support of this proposal whole cell patch-clamp recordings have revealed a small proportion of dissociated dorsal root ganglion cells from injured but not normal nerves that were spontaneously active within 24 hours of culture (Study & Kral, 1996). This study found that 18% of large diameter neurones 66% of medium diameter and 42% of small diameter neurones were spontaneously active. Kajander & Bennett recorded from individual primary afferent axons in microfilaments teased

from the dorsal roots (Kajander & Bennett, 1992; Kajander et al, 1992). They found that 35% of A beta, 15% of A delta and 3% of C fibres were spontaneously active. The rough correlation between cell size and fibre type (Harper & Lawson, 1985) suggests that fewer large diameter neurones but more medium and small diameter neurones were found spontaneously active by Study & Kral (1996) compared to the former study. The differences may be due to differences in the post-injury times, as Kajander and Bennett studied spontaneous activity at a later time period 1 to 3 days postoperatively as opposed to within six hours as well as differences in the recording conditions. At least some ectopic discharge in C fibres may originate in the soma as the spontaneous activity in these fibres has been reported to remain after the nerve was transected proximal to the injury site (Xie et al, 1995).

(bii) Generation of impulses at the neuroma

Spontaneous ectopic discharges have been reported to occur at the site of nerve injury between days 6-44 days postoperatively although this was not measured quantitatively (Xie & Xiao, 1990). Spontaneous activity has also been found in unmyelinated primary afferents after loose ligation of the saphenous nerve (Koltzenburg et al, 1994). In the study of Tal & Eliav (1996) electrophysiological recording from myelinated primary afferent axons revealed spontaneous impulse activity which originated at the site of nerve constriction. During the period 2-14 days postoperatively, 10% of the fibres sampled had spontaneous activity. The spontaneous activity fell into 3 patterns: tonic rhythmic pattern in which the interval between successive spikes in a train was uniform, ranging from 25-50ms (discharge rate 20-40Hz), interrupted bursty or "on-off" patterns with variable silent period between high frequency bursts and irregular ongoing pattern with random inter-spike intervals 5-15Hz. Axons trapped at the injury site, both those with spontaneous activity and those without, became hyper-excitable to mechanical stimulation. The

locus of mechanosensitivity shifted progressively in time from proximal end to the distal end of the injury site (Tal & Eliav, 1996).

2.6.1.(c) The time course of ectopic impulse generation

(ci) Complete transection

In transected nerves of the rat, ongoing activity originating from the neuroma begins 2-3 days postoperatively and reaches a maximum at 2 weeks (Wall & Gutnick, 1974; Govrin-Lippman & Devor, 1978). A peak incidence of spontaneous activity at the neuroma of approximately 25% occurred 10-14 days after transection (Devor 1994; Govrin-Lippmann & Devor, 1978). However, a comparable incidence of spontaneous activity (35% of the A beta and 15% of the A delta fibres) was present at 1-3 days postinjury with the Bennett & Xie model (Kajander & Bennett, 1992). Upon reaching a maximum, ectopic impulses from the neuroma rapidly decreased to approximately 4% of fibres sampled and remained at this level for at least another 190 days (Govrin-Lippman & Devor, 1978).

There is evidence that spontaneous activity develops first in myelinated fibres and later in unmyelinated fibres (Govrin-Lippmann & Devor, 1978; Janig, 1988; Devor, 1994). The incidence of evoked discharge in C-fibres begins much more slowly. Initially these fibres are silent after transection and spontaneous firing does not peak until about one month after the injury (Devor, 1994; Devor & Govrin-Lippmann, 1985).

(cii) Partial nerve injury

Increased ongoing activity in dorsal horn neurones has been reported as early as one hour following loose ligation of the sciatic nerve (Sotgui et al, 1994). This post injury discharge could be blocked by applying local anaesthetic to the

constriction site indicating that the source of the peripheral drive was the injury site (Sotgui et al, 1994). At 1 to 3 days after surgery, the proportion of fibres with spontaneously firing fibers originating in the dorsal root ganglia has been reported as 20 % (Kajander & Bennett, 1992), with most of the activity occurring in the A beta and A delta fibres. However, only a small percentage of the A beta and A delta axons with spontaneous discharges were still able to conduct impulses through the injured site (Kajander & Bennett, 1992). Abnormal spontaneous activity was found to peak at 6-9 days postoperatively in the dorsal root ganglia (Tal & Eliav, 1996). During this peak period an average of 13% of all A fibres fired spontaneously in these rats. Spontaneous impulses in large diameter afferents continues for at least several weeks postinjury (Xie & Xiao, 1990; Eliav & Tal, 1994; Xie et al, 1995).

Spontaneous C fibre discharge has been found to be essentially absent from the dorsal root ganglion cells during the first 3 days after loose ligation of the sciatic nerve (Kajander & Bennett, 1992). C fibre spontaneous discharge after transection is also very rare initially (Devor, 1994). As discussed previously, C-fibre discharge has been demonstrated in the later stages of the constriction-evoked syndrome (Xie & Xiao, 1990).

2.6.1.(d) Comparison of spontaneous activity after transection and partial nerve injury of the sciatic nerve

After peripheral nerve transection, the activity originating at the injury site is primarily fast, regular and continuous or has a bursting pattern whereas that from the dorsal root ganglia is predominantly a slower, random activity (Wall & Devor, 1983, Burchiel, 1984a). Spontaneous activity with highly irregular interspike intervals is the predominant mode of spontaneous activity from the dorsal root ganglia in vivo after peripheral nerve injury (Xie et al, 1995). An irregular pattern (with random inter-spike intervals) was more common at the site of injury after peripheral nerve injury

than after transection (Tal & Eliav, 1996). This occurred in 41% of fibres at the loose ligation site as compared to <5% in neuromas at 3-16 days postoperatively (Tal & Eliav, 1996).

2.6.1.(e) Mechanisms of ectopic impulse generation

It has been suggested that the accumulation of sodium channels could account for the ectopic discharges found after nerve injury as intense immunolabelling of sodium channels has been demonstrated at the site of nerve transection in the fish (Devor et al, 1989). In the rat, sodium channels in membranes of normal peripheral nerve axons and those following peripheral nerve transection have been investigated (Devor et al, 1993). Accumulation has been found at the neuroma, especially in demyelinated axons and end bulbs. From computer simulations of the repetitive firing process it has been found that the addition of extra Na^+ channels with no other change in active or passive membrane properties is sufficient to render an axon hyper-excitable and even to shift it from a state of silence to a state of spontaneous firing (Matzner & Devo, 1992). The action potential threshold has been found to be substantially more negative in spontaneously active neurones compared with controls (Study & Kral, 1996). The intrinsic threshold for rhythmic firing has been shown to be reduced at locations of sodium (Na^+) channel accumulation (Matzner & Devor, 1992).

Pharmacological agents that exhibit use-dependent block of Na^+ channels such as carbamazepine and phenytoin have been found to suppress neuroma discharge (Yaari & Devor, 1985; Burchiel, 1988; Chabal et al, 1989; Devor et al, 1992). Lidocaine molecules also bind to sites associated with voltage sensitive sodium channels and prevent channel opening in response to stimuli which depolarise the cell membrane. Systemic application of lidocaine has been found to suppress spontaneous activity generated at the neuroma and in the dorsal root ganglion cells in rats with a

sciatic nerve transection (Devor et al, 1992). The dose effective at blocking ectopic discharge was lower than that required to block the initiation or propagation of impulses by electrical stimulation (Devor et al, 1992). The dorsal root ganglia has been found to be more sensitive than the injury site to systemic lidocaine (Devor et al, 1992). This may be due to a difference in the intrinsic pacemaker encoding properties of the two ectopic impulse generating sites. As cited previously, there is evidence that neuroma afferents fire rhythmically at high frequency, while dorsal root ganglion cells fire slowly and irregularly (Wall & Devor, 1983).

Application of potassium channel blockers to the injured region of the nerve was without effect which was not unexpected as potassium channel blockers have been little if any effect on normal myelinated primary afferent axons (Kajander et al, 1992). However, potassium channel blockers such as 4-aminopyridine and gallamine applied to the dorsal root ganglion cells increased the frequency of spontaneous discharge or initiated activity from silent fibres (Kajander et al, 1992). Intravenous gallamine has been shown to increase the frequency of spontaneous activity in 50% of A beta and 15% of A delta fibres (Kajander & Bennett, 1992). Although no studies of changes in calcium channels after peripheral nerve injury have been reported, verapamil, an L-type calcium channel blocker, blocked spontaneous action potential activity when applied to the injury site (Xie et al, 1993).

2.7. ALTERED CENTRAL PROCESSING

The previous discussion has dealt with changes in the periphery. There is however evidence for altered central processing of afferent information particularly in the spinal cord. Mechanical allodynia present in some neuropathic patients has been found to be mediated by impulses in large myelinated afferents (A beta low-threshold mechanoreceptors) that normally convey innocuous tactile sensations (Wallin et al, 1976; Loh & Nathan, 1978; Campbell et al, 1988; Price et al, 1989; Gracely et al, 1990; Ochoa & Yarnitsky, 1993). It has been suggested that touch-evoked pain is the consequence of an abnormal central processing of A beta fibre input (Price et al, 1989; Gracely et al, 1992; Koltzenburg et al, 1992; Treede et al, 1992) although the mechanisms involved are uncertain. A number of studies have examined whether altered central processing contributes to the behavioural syndrome in the rat model and to neuropathic pain in man.

2.7.1. CLINICAL AND BEHAVIOURAL STUDIES

Cold hyperalgesia which has been reported in some patients may be due to abnormal central processing of input from normal cold specific afferents impinging on sensitised central neurones (Fruhstorfer & Lindblom, 1984). Torebjork and co-workers however found that block of myelinated fibre conduction did not prevent heat hyperalgesia and suggested that its aggravation by noradrenaline may reflect sensitisation of the nociceptors themselves rather than alterations in central neuronal processes conveying pain (Torebjork et al, 1995).

Supporting these observations in humans are studies in animal models of partial nerve injury. The associated abnormal behaviours which develop after injury in these animal models such as thermal hyperalgesia may be due to an over excitation of dorsal horn cells (Davar et al, 1991; Mao et al, 1992a & b; 1993; Seltzer et al, 1991;

Tal & Bennett, 1993; Yamamoto & Yaksh 1992). If this is correct then modifying the hyper-excitability of spinal dorsal horn neurones induced by injury discharge could reduce the pain associated behaviours characteristic of the neuropathic model. It is also possible that pre-emptive treatment before surgery which reduced or abolished the injury discharge might modifying the development of excitability in the dorsal horn neurones and thus prevent the abnormal behaviours from developing in the Bennett & Xie model. This has been investigated by a number of pharmacological procedures.

2.7.1.(a) Local anaesthetics

Dougherty et al (1992) found a reduction in both the duration and magnitude of the heat hyperalgesia in neuropathic animals which had local anaesthetic applied to the wound for 5 minutes immediately following ligation of the nerve. Although this study found that injury related discharge is an important factor in the generation of hyperalgesia in the Bennett & Xie model, the Seltzer model did not show this sensitivity. Bupivacaine applied to the sciatic nerve before the skin incision and around the nerve for 10 minutes before ligation delayed the onset of thermal changes until day 7 postoperatively. When given 15 minutes after the nerve constriction injury, bupivacaine had no effect on the development of thermal "hyperesthesia" (Yamamoto et al, 1993). They also showed that systemic bupivacaine had no effect on the development of thermal hyperesthesia. Sotgiu and co-workers, pre-treated animals either by applying lidocaine to the nerve for 10 minutes or by systemic injection of this local anaesthetic for 5 minutes before surgery. They found that the lidocaine prevented the development of thermal hyperalgesia and postulated that this was due to blocking the early afferent injury discharge (Sotgiu et al, 1995b).

Lidocaine has also been found to decrease the baseline activity during the block when given after nerve injury (Sotgiu et al, 1996). In complete nerve transection studies, systemic lidocaine given postoperatively suppressed the ectopic

impulses generated both at the site of nerve transection and in axotomised dorsal root ganglion cells (Devor et al, 1992). The local anaesthetics, lidocaine, tocainide and mexiletine were found to reduce the spontaneously active fibres originating in the sciatic neuroma seven days after injury (Chabal et al, 1989). No conduction block occurred with these intravenous doses.

Clinically, lidocaine has been found to produce a transient effect on neuropathic and postoperative pain (Cassuto et al, 1985; Kastrup et al, 1987; Bach et al, 1990). Lumbar epidural blocks of bupivacine and morphine 72 hours before surgery were shown clinically to prevent phantom limb pain in 3 patients (Bach et al, 1988).

2.7.1.(b) NMDA receptor antagonists

There is evidence that L-glutamate is a major excitatory transmitter released from the central terminals of primary afferents and that in normal animals the action of this excitatory amino acid is blocked by the antagonist activity at non-NMDA receptors (Curtis et al, 1959; Bernardi et al, 1972; Duggan, 1974; Dickenson & Sullivan 1986; Woolf & King 1987; Ma & Woolf 1995). When inflammation develops peripherally, glutaminergic transmission within the spinal cord increasingly involves NMDA receptors (Schaible et al, 1991; Wilcox, 1991; Woolf & Thompson, 1991; Ren et al, 1992). Thus not surprisingly, the role of NMDA receptors has been investigated in relation to neuropathic pain as the activation of these receptors may lead to sustained depolarisation of local circuit and second order dorsal horn neurones (Dubner & Ruda, 1992).

Both pre-emptive studies and post-injury studies using NMDA antagonists have been performed in the Bennett & Xie model. Beginning 15 minutes prior to ligation, four daily injections of MK-801, a non competitive NMDA receptor antagonist, were found to reduce thermal hyperalgesia when administered

intrathecally (Mao et al, 1992a) or or via i.p. injections (Mao et al, 1992b). This was also found to occur when HA966, a non-competitive NMDA antagonist acting at the glycine site was given over the same time course intrathecally (Mao et al, 1992a). MK-801 injected systemically (i.p.) 30 minutes prior to and seven days following nerve ligation prevented the development of thermal hyperalgesia over the 10-37 day postoperative period tested (Davar et al, 1991). Pre-emptive treatment with subcutaneous MK-801 given 30 minutes prior to and twice daily for 8 days following loose ligation prevented the development of mechanical hyperalgesia measured 27 days later (Smith et al, 1993). Memantine, another NMDA antagonist, significantly reduced thermal hyperalgesia induced by partial nerve injury for up to 14 days following 7 days i.p. administration by osmotic pump before surgery. This study, found that in a separate group of animals which had not been treated before surgery, memantine, administered (i.p.) 7 days or 14 days postoperatively reversed the existing hyperalgesia for 1 hour (Eisenberg et al, 1995).

Thermal hyperalgesia was also found to be reduced for at least 48 hours by a single injection (i.p. or i.t.) of MK-801 given 3 days after ligation. Intrathecal MK-801 was without effect upon the response latency of the normal or sham operated paw but selectively reversed the thermal hyperalgesia when administered intrathecally 7 days after nerve injury (Yamamoto & Yaksh, 1992). Intrathecal administration of dextrorphan , another non competitive NMDA antagonist, 8-10 days postoperatively reduced the heat evoked hyperalgesia (Tal & Bennett, 1993).

There is also clinical evidence for the involvement of NMDA receptors in the spinal generation of neuropathic pain (Backonja et al, 1994; Kristensen et al, 1992; Max et al, 1995). The NMDA antagonist, CPP, was administered intrathecally to one patient with neurogenic pain (Kristensen et al, 1992). Although the spread of the pain was reduced, anxiety and nightmares occurred 4 hours after injection. In six patients with neuropathic pain syndromes studied by Backonja et al (1994) and eight

patients with chronic pain and widespread allodynia (Max et al, 1995) symptomatic relief was accompanied by unpleasant side effects.

2.7.1.(c) Alpha two adrenoceptor agonists

Yamamoto & Nozaki-Taguchi (1996) found that intrathecal clonidine, an alpha 2 adrenoceptor agonist, administered before nerve injury significantly delayed the onset of thermal hyperesthesia by 3 days. This effect of clonidine was antagonised by co-administration with idazoxan, an alpha 2 antagonist. Postoperatively, this study found that clonidine had no effect on the development of hyperalgesia. They suggested that the delay in the onset of thermal hyperesthesia was due to clonidine decreasing the sympathetic outflow from the spinal cord.

The development of hyperalgesia between 26 & 29 days following nerve injury using the Bennett & Xie model was prevented by administering clonidine 30 minutes before and 6 hours after surgery (Smith et al, 1993). In animals pre-treated with clonidine the paw withdrawal threshold of the ipsilateral paw was not significantly different from the threshold obtained for the contralateral paw. This group suggested that clonidine prevented the development of hyperalgesia by inhibiting the release of primary afferent neuropeptide transmitters such as substance P and CGRP and depressing the response of dorsal horn neurones (Kuraishi et al, 1985; Go & Yaksh, 1987). An action in the spinal cord was suggested due to the presence of a high density of alpha 2 adrenoceptor binding sites in Lamina II of the dorsal horn (Young & Kuhar, 1979; 1980) which is the termination area of nociceptive C-fibres (La Motte, 1977; Light & Perl, 1979).

Xu et al (1993b) investigated the effect of intrathecal guanfacine (alpha 2a adrenoceptor selective agonist) & clonidine (non-selective alpha 2 adrenoceptor agonist) on the flexor reflex in rats with intact & sectioned sciatic nerves. Both

guanfacine and clonidine dose dependently depressed the flexor reflex in rats with intact nerves. However, 4 to 18 days after sciatic nerve section there was a dramatic increase in the sensitivity of this reflex only to the depressive effect of i.t. clonidine. Xu and co-workers suggested that the analgesic effect of clonidine was not mediated solely by alpha 2a adrenoceptors since clonidine depressed the flexor reflex with stronger efficacy than guanfacine. Clonidine has a similar affinity for alpha 2a and alpha 2c adrenoceptors (Uhlen et al, 1992) which suggests that both adrenoceptors subtypes were involved in the depressive effect. Yaksh et al (1995) investigated the effect of alpha 2 agonists in the spinal cord on mechanical allodynia induced by the Chung model. They found that lumbar injection of alpha-2 but not alpha-1 resulted in a dose-dependent reversal of the ligation induced allodynia, thus supporting a role for spinal adrenoceptors in producing the anti-allodynic effects (Yaksh et al, 1995).

2.7.1.(d) morphine

In normal rats and cats, morphine has been found to depress the stimulus-evoked activity of nociceptive dorsal horn neurones (Duggan et al, 1976; Le Bars et al, 1975; Lombard & Besson, 1989). Thus, pre-emptive administration of morphine before nerve injury by the Bennett & Xie method might prevent the spinal dorsal horn neurones from becoming hyper-excitable. However, Yamamoto & Nazaki-Taguchi (1996) found that pre-emptive morphine administered i.t. 20 minutes before surgery had no effect on the development of thermal hyperalgesia which occurs after peripheral nerve injury.

In contrast, most studies using the Bennett & Xie model found that morphine reduced hyperalgesia when administered after the surgery producing nerve injury (Attal et al, 1991; Jazat & Guilbaud, 1991; Yamamoto & Yaksh, 1992; Yamamoto et al, 1994; Kayser et al, 1995). Morphine given intrathecally 1 week after nerve injury, resulted in a dose dependent effect by reducing the thermal

hyperalgesia associated with this model (Yamamoto & Yaksh, 1992; Yamamoto et al, 1994).

Intravenous morphine administered 2 weeks after injury resulted in a decrease in the vocalisation threshold for paw pressure which was maximal 15 minutes after injection lasting 20-25 minutes in total (Attal et al, 1991). Two weeks after nerve injury, intravenous morphine was found by Kayser et al (1995) to increase the mechanical threshold to paw pressure and by Jazat & Guilbaud (1991) to induce a prolonged decrease in the pain rating score. In contrast, another study found that i.t. morphine given 8 days after injury produced significant anti-noiception in sham operated but not in nerve injured rats with established thermal hyperalgesia (Mao et al, 1995). Using the Kim & Chung model intrathecal morphine has been found to have no effect on mechanical allodynia (Bian et al, 1995).

It should be noted that neuropathic pain in man does not always respond well to morphine. Relatively high doses are needed which are often associated with significant side effects (Portenoy & Foley, 1986; Arner & Meyerson, 1988; Portenoy et al, 1990).

2.7.2. CENTRAL STRUCTURAL ALTERATIONS

2.7.2.(a) Transynaptic degeneration

The Bennett and Xie model of loose ligation of the sciatic nerve has been found to result in the presence of dark neurones in laminae I-IV in both sides of the lumbar spinal cord (Sugimoto et al, 1989; Sugimoto et al, 1990). These neurones are pyknotic and hypochromatic and have been described as dark neurones because the staining densities of their cytoplasm and nucleoplasm has been elevated (Sugimoto et al, 1984). Many have been shown to be smaller than normal and appear shrivelled which suggests that they have been damaged. These neurones increased only in the

lumbar dorsal horn and were reported to be statistically higher in the spinal cord ipsilateral to the nerve injury (Sugimoto et al, 1989; Sugimoto et al, 1990). They have been found to be concentrated in the medial two thirds of laminae I-II which corresponds to the sciatic nerves intraspinal terminal field (Devor & Claman, 1980; Swett & Woolf, 1985). The remaining area, the lateral one third of laminae I-II not innervated by the damaged nerve (Devor & Claman, 1980; Swett & Woolf, 1985), displayed no increase in the number of dark neurones after ligation of the sciatic nerve (Sugimoto et al, 1989; Sugimoto et al, 1990). The number of dark neurones produced by the Bennett and Xie model has been found to be enhanced in rats which received strychnine (Sugimoto et al, 1989). In contrast, dark neurones were found after transection of the inferior alveolar nerve only in rats that received strychnine. These changes were most clearly evident 3-4 weeks postoperatively (Sugimoto et al, 1986; Sugimoto et al, 1987a). This perhaps explains why transection of the sciatic nerve of the rat failed to evoke transynaptic degeneration when investigated eight days after injury (Sugimoto et al, 1990).

Two mechanisms have been suggested to explain the presence of dark neurones after chronic constriction injury. Operations where the nerve has been manipulated but not damaged have found that two days after these bilateral sham operations of the sciatic nerve, dark neurones were present in laminae I-III bilaterally (Nachemson & Bennett, 1993). Bilateral nerve surgery with nerve manipulation was found to produce a greater effect than bilateral surgery without nerve manipulation. The increase on each side of the cord in the Bennett and Xie model was significantly less than the increase seen in either bilateral group as the surgery is performed only on one nerve with this model. It was suggested by this group that nociceptive input arising from the surgery produces an initial change in neurones which was detectable for a only a few days after surgery and decreases when the tissue injury has healed.

It is possible that ectopic discharge is the second mechanism involved in the production of dark neurones in the Bennett and Xie model (Sugimoto et al, 1990,

Nachemson & Bennett, 1993). Supporting this, it has been proposed that high levels of ectopic discharge in damaged primary afferents may be critical for the production of transynaptic degeneration that follows alveolar nerve transection (Sugimoto et al, 1987a, Sugimoto et al, 1987b). It has been hypothesised that dark neurones are the result of an excitotoxic insult due to excess release of glutamate (Nachemson & Bennett, 1993; Krogsgaard-Larsen, 1992).

It has also been speculated that at least some dark neurones are inhibitory interneurones. The strychnine-enhanced presence of dark neurones may be due to excessive excitation that is exacerbated by strychnine induced disinhibition (Sugimoto et al, 1989). Supporting this, glycine inhibition by intrathecal strychnine in normal rats produces allodynia (Yaksh, 1989). The functional impairment or death of these neurones will result in altered processing of afferent information and may contribute to a central hyper-excitable state relevant to the behavioural syndrome (Nachemson & Bennett, 1993).

2.7.2.(b) Sprouting

It has been reported that large sensory axons which normally terminate in laminae III and below sprout into laminae II after peripheral nerve injury (Lekan et al, 1996). It has been hypothesised that these sprouting fibres make synaptic connections with post-synaptic cells which normally process nociceptive information. Thus sprouting may contribute to touch -evoked allodynia which occurs following nerve injury. Sprouting will be discussed in relation to the immunoreactive NPY changes that occur after peripheral nerve injury in section 6.3.(g).

2.7.3. ELECTROPHYSIOLOGICAL PROPERTIES OF SPINAL NEURONES

2.7.3.(a) Spontaneous activity and afterdischarges

Using extracellular recordings in anaesthetised animals, Laird and Bennett (1993) sampled dorsal horn neurones of the lumbar enlargement. This study included low threshold mechanoreceptors which repond to light touch, wide dynamic range neurones (WDR, responding to innocuous and noxious stimulation) and nocieptive specific neurones which respond to high intensity stimulation. They found the majority of neurones (77%) in both the sham-operated and in the nerve-injured rats had no background activity. In the nerve injured animals 16% of the spontaneously active neurones had spontaneous firing rates <7Hz and 7% of had firing rates >7Hz as opposed to 22% of cells in the sham animals having firing rates <7Hz and 1% having firing rates >7Hz (Laird & Bennett, 1993). They found the mean rate of spontaneous firing in the 7% of cells in the nerve-injured animals was approximately 26Hz ranging from 10-60Hz (Laird & Bennett, 1993). Sotgiu and co-workers (1992, 1995) found that the background activity of WDR neurones on the side ipsilateral to the nerve injury ranged from 18 to 27Hz which was significantly higher than that of spinal neurones recorded on the contralateral side (Sotgiu et al, 1992; Sotgiu 1995). Systemic lidocaine was found to reduce the background activity of these neurones on the ipsilateral side but had no effect on the normal spontaneous activity of neurones of the contralateral side (Sotgiu et al, 1992).

Afterdischarges can be defined as the persistence of a response after the termination of the stimulus. It has been suggested that afterdischarges of spinal neurones may contribute to the exaggerated behavioural responses (Sotgiu et al, 1995a). In rats with chronic constriction injury of the sciatic nerve, long lasting afterdischarges have been described in large populations of neurones in the dorsal horn (Palecek et al, 1992b; Laird & Bennett, 1993; Sotgiu et al, 1993), thalamus and

cortex (Guilbaud et al, 1990). After discharges have been reported to increase after nerve injury (Laird & Bennett, 1992; Palecek et al, 1992b; Sotgiu et al, 1995a). Sotgiu and co-workers have found that the duration and magnitude of the afterdischarges differed significantly depending on the type of neurone (Sotgiu et al, 1995a). Neurones responding to noxious mechanical stimuli had afterdischarges with durations of 8-65 seconds compared to nociceptive specific neurones which had durations of 6-20 seconds (Sotgiu et al, 1995). Laird & Bennett (1993) found that thirteen percent of dorsal horn neurones displayed abnormal responses such as prolonged afterdischarges to very brief innocuous mechanical stimuli and high activity in the absence of any intentional stimulation. These observations suggest enhanced mechanosensitivity in a fraction of spinal neurones that may be associated with mechanical allodynia following nerve injury.

A sustained increase in background activity as a result of prior stimulation of the receptive field or of electrical afferent stimulation was found in 5% of dorsal horn neurones in sham and with 13% of neurones of nerve injured animals (Laird & Bennett, 1993). In addition, all of these neurones were activated by tapping the nerve-injury site (Laird & Bennett, 1993). In contrast, Takaishi and co-workers did not find prolonged responses or increased spontaneous activity in spinal wide dynamic neurones using the Seltzer model of ligation in rats (Takaishi et al, 1996). Palecek et al, (1992a) found increased levels of spontaneous discharge in spinothalamic tract neurones of primates with an adaptation of the Kim & Chung model whereby the spinal nerve L7 was ligated.

2.7.3.(b) Responses to mechanical stimulation

Laird & Bennett (1993) studied the receptive field properties of dorsal horn neurones in the lumbar enlargement of the spinal cord at 9-11 days after peripheral nerve injury using the Bennett & Xie model. Fifty six percent of the neurones excited

by electrical stimulation proximal to the nerve constriction had no detectable mechanical receptive field (within the normal innervation territory of the sciatic nerve or any of the remainder of the body accessible to stimulation) which is indicative of partial deafferentation. Activity could be evoked by electrical stimulation of the sciatic nerve at an intensity sufficient to excite C fibres in the majority of neurones with a low threshold mechanical receptive field. This corresponds with the relatively greater number of unmyelinated fibres that survive the nerve injury (Basbaum et al, 1991; Carlton et al, 1991; Munger et al, 1992). No statistically significant differences were found between mean size of the receptive fields between sham-operated and nerve injured rats in neurones with the same input properties (Laird & Bennett, 1993). In a study by Takaishi and co-workers (1996) mechanical receptive field areas in the 5-week post-ligation animals were not significantly different from those obtained in sham animals. In contrast, at 16 weeks after injury the receptive field areas in both the ipsilateral and contralateral side of the nerve injured rat were significantly greater . These changes did not occur quickly enough to account for the rapid onset of behavioural hyperalgesia and allodynia in these animals. Laird & Bennett (1993) found that the mean mechanical threshold necessary to excite cells (measured with Von Frey hairs) in the sham operated animals did not differ from the mean threshold of cells measured after injury. However, the proportion of dorsal horn neurones excited only by low-intensity stimulation of their receptive field was lower in the nerve-injured than in sham-operated animals (Laird & Bennett, 1993). This agrees with the results obtained from recording from primary afferent axons (Kajander & Bennett, 1992) and anatomical studies which have shown that the large myelinated fibres which carry information from the low-threshold mechanoreceptors are preferentially affected (Basbaum et al, 1991; Carlton et al, 1991; Munger et al, 1992). The difference between the thresholds measured in deep neurones in the sham-operated and in nerve-injured rats was statistically significant with the nerve injured rats having a higher threshold (Laird & Bennett, 1993).

Tapping the neuroma gently evoked a response in 53% of the cells in the nerve injured animals but tapping the sciatic nerve at a comparable position did not produce a response in any of the neurones recorded in the sham-operated animals (Laird & Bennett, 1993). The majority of neurones with a response to tapping the neuroma had a C-fibre input. In a few dorsal horn cells the effect of extending the leg which stretched the nerve and moved the neuroma was found to evoke a sustained discharge (Laird & Bennett, 1993). Similarly, some of the WDR neurones ipsilateral to the nerve injury responded to mechanical probing around the nerve ligation in the Seltzer model (Takaishi et al, 1996).

2.7.3.(c) Responses to thermal stimulation

Even though rats display thermal hyperalgesia following a sciatic nerve partial injury, two studies have reported no alteration in the responses of spinal neurones to noxious thermal stimuli (Palecek et al, 1992b, Laird & Bennett, 1993). In contrast enhanced neuronal responses to noxious heat has been reported on the injured side in monkeys after an L7 spinal nerve tight ligation (Palecek et al, 1992a). Takaishi et al (1996) hypothesised that in rats exhibiting behaviour of heat hyperalgesia and allodynia, dorsal horn neurones ipsilateral to the ligation should show enhanced responsiveness to noxious heat and have enlarged receptive fields, compared to neurones on the non-ligated side or from sham-operated rats. Contrary to this hypothesis they found no statistically significant changes in the stimulus-response functions of dorsal horn neurones evoked by graded noxious heat. Laird and Bennett reported that 69% of nerve-injured animals responded to a noxious heat stimulus. A response to innocuous cold stimuli was found in 2/33 neurones tested in sham-operated animals and in 2 of the 30 neurones tested in the nerve-injured animals. In addition, these cells all had C-fibre input (Laird & Bennett, 1993). It therefore remains unclear whether enhanced neuronal thermo-sensitivity underlies heat hyperalgesia in partial nerve injury models.

2.8. PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE

I shall discuss details of the preparation and use of antibody microprobes in several sections of this thesis. As an introduction however it is appropriate to outline the principles of this method and its limitations.

The antibody technique was developed

"as a means of getting better spatial resolution in determining sites of release of neuropeptides in the central nervous system and to do so with minimal disturbance to the structures giving release" (Duggan, 1992).

Antibody microprobes were developed by Duggan & Hendry (1988) and is currently the least damaging technique available for the in vivo detection of peptide release within the central nervous system. It has been estimated that sites of release of 100μm diameter can be detected with this technique (Duggan, 1991). Microprobes are modified glass micropipettes of similar diameters to those used as microelectrodes for extracellular recordings in electrophysiological studies. The tip size of each microprobe is 5-10μm and thus damage to the preparation is kept to a minimum. This is in contrast to diameters of 300-500μm when microdialysis and push-pull cannula techniques are employed (Philippu, 1984; Ungerstedt, 1984).

Microprobes bind molecules from the extracellular space. The detection of bound ligand (the endogenous peptide) can be done in a number of ways. Ideally a second antibody which will bind to the bound peptide could be used. Thus an antibody to the N-terminus or mid-portion of a ligand would be immobilised on microprobes and a C-terminus antibody, which has been radiolabelled, subsequently used to locate bound peptide molecules. Two site assays have been unsuccessful however with substance P (11 amino acids) presumably because of steric hindrance. Therefore more commonly the bound peptide has been detected by the subsequent failure of binding of radiolabelled peptide molecules.

Briefly, antibody microprobes are fine glass micropipettes, which have a siloxane coating bearing free amine groups. Glutaraldehyde, a cross linking molecule, reacts covalently with the free amine groups of the coating and the amine groups of protein A. Protein A is bound to the siloxane coating to immobilise the antibodies of the neuropeptide of interest. Microprobes are inserted into the spinal cord or brain and left *in situ* for a defined period of time so that a proportion of the released endogenous peptide binds to a restricted area of the adjacent microprobe surface. Following removal from the CNS, the microprobes are incubated with a radiolabelled form of the peptide. The microprobe is then washed and placed on X-ray film to obtain an autoradiographic image. Binding of the endogenous peptide is detected as the failure of regions of the microprobe to bind the radiolabelled form of the peptide which are analysed quantitatively with a computer assisted image analysis system. The distribution of the amounts of bound radiolabelled peptide is determined by changes in the image density (or grey scale value) along the length of the autoradiographs with any deficits in the tracer binding being represented graphically as comparatively low grey scale values. A sorting program can select groups of microprobes according to stimulus /experimental parameters and prepare a mean image analysis. In addition, pairs of such grouped data can be compared and the significance of their regional differences evaluated statistically. The release pattern of an endogenous ligand detected on the antibody microprobes can be related to histology and hence to the laminae of the spinal cord or to discrete areas of the brain.

The antibody technique has been employed in the spinal cord to study the release of:

B-endorphin in the cat brain (Williams et al, 1994b) and rat brain (Duggan et al, 1993)

calcitonin gene related peptide in the cat (Morton & Hutchison, 1989, 1990; Schaible et al, 1994) and the rat (Schaible et al, 1994)

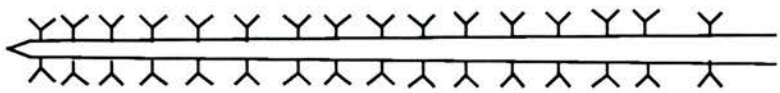
dynorphin in the cat (Hutchison et al, 1990) and in the rat (Riley et al, 1996)

Figure 2. Principles of the antibody microprobe technique for the *in vivo* detetection of neuropeptide release I.

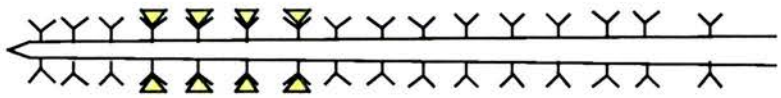
This shows the principles from antibody immobilisation on the outer surface of the microprobes to the production of autoradiographic images.

PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE I

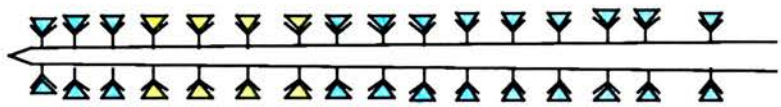
1. Antibodies (Y) to a neuropeptide of interest are immobilised onto the outer surfaces of glass micropipettes.



2. Locally released neuropeptide (▽) binds to adjacent antibodies *in vivo*.



3. Radiolabelled form of the peptide (▽) in which microprobes are subsequently incubated *in vitro* binds to free antibody sites.



4. After washing microprobes are placed on X-ray film to obtain an autoradiographic image.

5. Autoradiographs indicate sites of extracellular neuropeptide presence.

Area of reduced image = area of reduced radioligand binding
= area of endogenous ligand binding

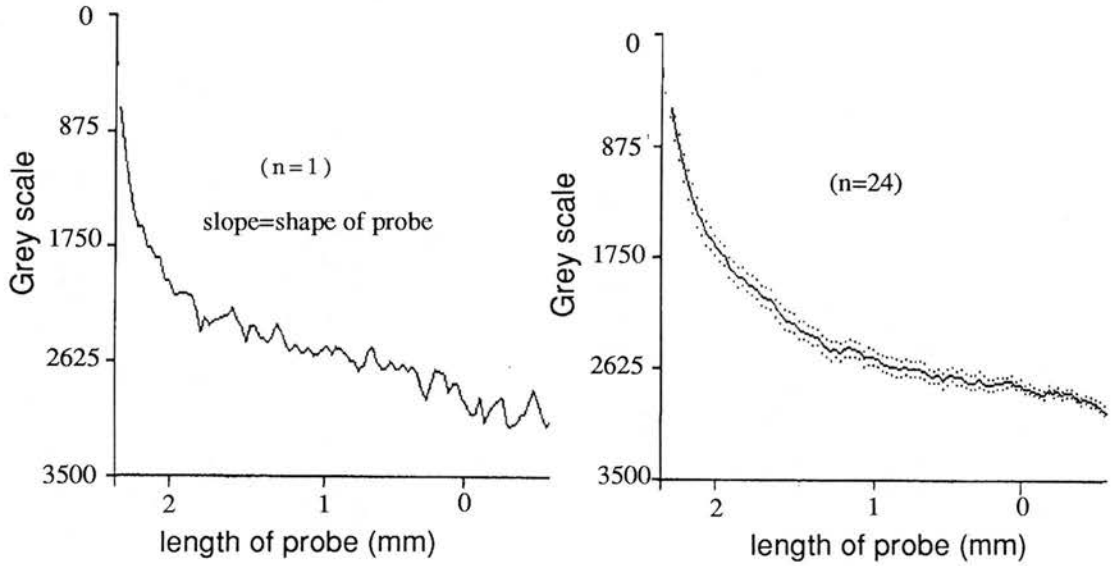
Figure 3. Principles of the antibody microprobe technique for the in vivo detection of neuropeptide release II.

Image analysis of the autoradiographic images reveals sites of endogenous peptide binding.

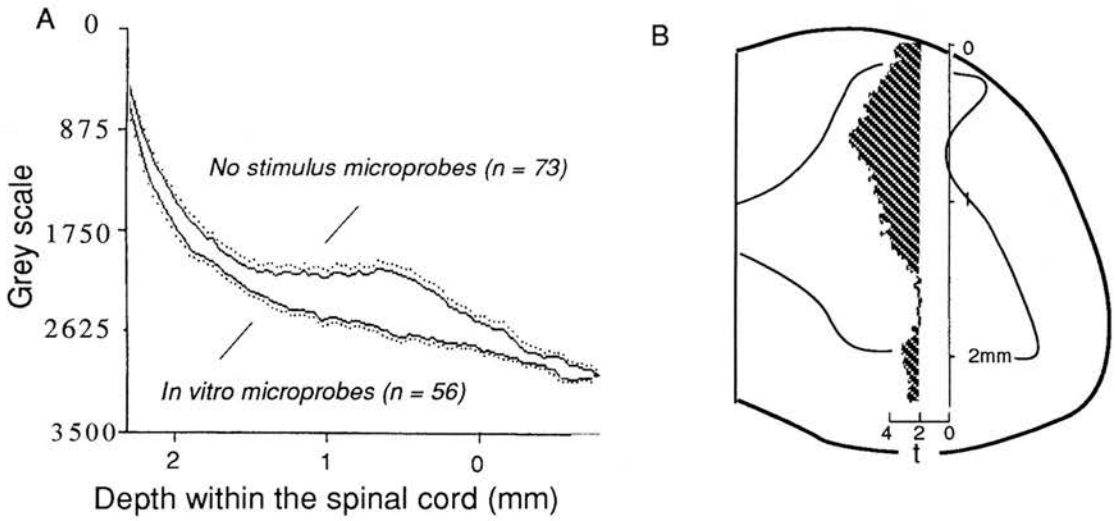
PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE II

1. Using a computer-assisted image analysis system, autoradiographs are converted into plots of image density (or grey scale values) v.s. length at 30μm intervals. The analysis system allows data from a group of microprobes to be pooled and a plot of mean image density (+/- s.e.m.) at each 30μm interval to be produced (A). The means are joined to give a continuous line but the s.e.m. at each analysis point is plotted separately.

in vitro microprobes



2. Mean image analysis for two groups of microprobes can be statistically compared at each 30μm interval, using multiple Students' t-tests (A). Areas of statistically significant differences (i.e. where t>2, P<0.05) in level of neuropeptide detection can be referred to depth within spinal cords (B).



enkephalins in the cat brain (Williams et al, 1992)

galanin in the spinal cord of the cat (Morton & Hutchison, 1989) and the rat (Hope et al, 1994)

neurokinin A in the cat spinal cord (Duggan et al, 1990, 1991b, Hope et al, 1990 a;b; Lang et al, 1991); and in the rat brain (Furmidge et al, 1995)

neuropeptide Y in the cat and rat spinal cord (Mark et al, 1997) and in the cat brain (Williams et al, 1993)

somatostatin in the cat spinal cord (Morton et al, 1989)

substance P in the spinal cord of the cat (Duggan & Hendry, 1986; Duggan et al, 1987; 1988a; b; 1991a; 1992, 1995, Hutchison & Morton, 1989; Morton et al, 1990; Schaible et al, 1990; 1992; Zhao et al, 1992; Lang et al, 1994) and rat (Lang & Hope, 1994); and in the brain of the cat (Williams et al, 1994a) and rat (Furmidge et al, 1993).

Thyrotrophin releasing hormone in the rat brain (Waterfall et al, 1993;1994)

The amount of time the microprobes need to remain in the CNS to detect the endogenous peptide varies according to local neuropeptide concentrations and the sensitivity of the particular microprobes, ranging in these previous studies from 5 to 30 minutes. A recent study showed that 150 stimuli delivered at 0.5Hz for a 5 minute duration, resulted in just detectable release of ir -SP in the spinal cord by microprobes (Duggan et al, 1995).

Microprobes detect the basal presence of a peptide by comparing the mean image analysis of a number of microprobes present in the spinal cord in the absence of any active peripheral stimuli with that of a group of *in vitro* microprobes not inserted into the spinal cord but incubated in radiolabelled peptide and processed concurrently with those used *in vivo*. To determine the sites at which this basal presence is significant the differences between these two groups are determined (usually in 30μm intervals) using the student's t-test. Significance between the two groups is obtained

when the difference between the two groups is large and the variance is small. Statistical significance indicates the probability that the peptide was detected (see figure 3).

Release studies commonly depend upon suppression of inactivation mechanisms to be successful as rapid inactivation of a peptide may make it difficult to collect released molecules. Collection depends on overflow or that a proportion of released molecules should escape rapid inactivation. Inactivation of neuropeptides is believed to be largely enzymatic (uptake has not been demonstrated) but there is a lack of information on the precise location of enzymes in relation to sites of release (McKelvey, 1986). Detection of release should not require the use of enzyme inhibitors if the neuropeptide is not rapidly inactivated following release. The term "volume transmission" has been used to describe the consequences of slow inactivation of released compounds (Fuxe et al, 1990). Fuxe & collegues propose that, at sites of release, low affinity receptors located at the releasing synapse are activated. If the released substance is not immediately degraded but is able to diffuse widely, the presence of high affinity receptors outside the releasing synapse effectively enables physiological effects to occur on neurones located over relatively large areas remote from sites of release. With such a mechanism, determining extracellular levels has more meaning in terms of predicting whether stimuli are adequate to produce receptor activation than the situation where overflow through suppressed inactivation is needed to detect release.

Microprobe experiments have attempted to define the peripheral stimulus producing release of the chosen neuropeptide after stimulation. The use of a range of stimulus types increases the likelihood that peptide release will be detected in instances where the physiological role of that peptide is not well understood and hence the relevant stimuli not well known. Antibody microprobes detect the extracellular presence of a peptide and a working definition of release is when a stimulus causes an increase in the extracellular levels of the peptide of study. This is

determined by comparing the mean image analysis of microprobes present in the spinal cord in the absence of any active peripheral stimuli with those present during stimulation. The finding of elevated levels of a neuropeptide after a peripheral stimulus has ceased has been interpreted as persistence and supports the suggestion that neuropeptides are slowly degraded. An example of this is neurokinin A. This neuropeptide appears to be relatively resistant to the enzymes believed to be important in the degradation of substance P (Nyberg et al, 1984; Hooper et al, 1985; 1987; Theodorsan-Norham et al, 1987). Substance P release in the cat spinal cord following noxious cutaneous stimulation or the development of peripheral inflammation has been found to be relatively focal in the upper dorsal horn whereas immunoreactive neurokinin A has been shown to persist and spread after release by the same stimuli (Duggan et al, 1990; Hope et al, 1990b).

It is usual to average the results from a number of microprobes. Although in normal cases it is clear that a defined stimulus has produced a localised intense deficit in the binding of a labelled peptide on single microprobes, this is not always the case. Particularly when dealing with a slowly degraded compound, relatively diffuse reductions in the binding of the tracer may not be apparent on single microprobe images and averaging the images from many microprobes becomes necessary to define differences between stimulus and non-stimulus groups. The differences between the mean image analysis of two groups indicates the regions over which a particular stimulus or treatment has produced release.

Although more of a qualitative technique, Morton and co-workers estimated the concentration of somatostatin to which the microprobes were exposed *in vivo,* by relating images of microprobes exposed to differing concentrations of the neuropeptide *in vitro* to those of microprobes that had been inserted into the spinal cord (Morton et al, 1989).

2.8.1. LIMITATIONS OF THE MICROPROBE TECHNIQUE

The microprobe technique is a form of radioimmunoassay in which the antibody has been immobilised. Unlike a conventional radioimmunoassay, microprobes do not allow the simultaneous competition between ligands for binding to available sites as unlabelled peptides are allowed first access. Therefore the antibody must have a sufficiently high affinity such that the radiolabelled peptide does not significantly displace the peptide within the period of incubation. Microprobes operate within the constraint of antibody specificity and hence cannot give exact chemical identity to what is detected. The identity of what is bound can be rendered only highly probable by other similar results with different antibodies recognising different sequences of the one polypeptide. C-terminally directed antisera are commonly used. The use of N-terminally directed antibodies is often limited by the unavailability of appropriately radiolabelled peptides. Schaible et al (1990) used both N-terminal and C-terminal directed antibodies and [I^{125}]-Bolton-Hunter SP and [I^{125}-Tyr^{8}]-SP in studying substance P release in the spinal cord of the cat. Hence it is possible that as well as detecting the full length peptide, shorter fragments resulting from enzymatic degradation might also bind to microprobes. Since extracellular compounds are being sampled it is unlikely that precursor molecules need to be considered.

Interpretation of results obtained with antibody microprobes also requires that other causes of reduced binding of the tracer be considered. Apart from the specific ligand, antibodies can bind non-specifically with other compounds. The amount of non-specific binding can be determined by in vitro studies and occurs only at a low level (usually 10-20% of total binding). Degradation of the antibody by proteases could occur following repeated penetration of the meninges of the brain during the third or fourth hour of microprobe insertion. This is a form of sterile inflammation but equally possible with prolonged experiments with the development

of infection. Protease degradation of antibodies should result in reduced binding of labelled $F(ab)_2$ fragments directly at the Fc component of the immobilised antibody at the portion of microprobe which had contact with the subarachnoid space and hence this control is used. Protease attack can be minimised by limiting the number of microprobes inserted into the brain and by irrigating the insertion site with autoclaved Ringers solution.

CHAPTER 3: General Methodology I: Surgical procedures

3.0. GENERAL METHODOLOGY I

3.1. SURGICAL PROCEDURES AND THE NEUROPATHIC MODEL

Male rats were purchased from Charles River Ltd (Margate, Kent, UK.) and were housed for at least two days prior to their experimental use in order to minimise any stress-induced alterations in their physiology that might be brought about by transportation and change of environment. They were housed in groups of 3 to 4 in standard rat cages on a 12 hour light/12 hour dark cycle, with room temperature of 19-23°C. Food and water were available *ad libitum.*

3.1.(a) Preparation of the rat neuropathic pain model

For the production of the Bennett & Xie model, adult male Wistar rats (200-350g) were anaesthetised with sodium pentobarbitone (50mg/kg i.p.). The midthigh area was shaved and washed with a solution of alcohol and Hibbiscrubb. Using full aseptic technique, an incision was made midthigh and the sciatic nerve exposed. Under low magnification, four 4/0 chromic gut loose ligatures were placed round the sciatic nerve, proximal to its trifurcation, and tensioned so that they were just unable to move up or down the nerve. This was found to barely arrest circulation. In the sham group, the right sciatic nerve was exposed and manipulated but not ligated. The muscle layer was closed and then the skin using subcuticular stitching with Vicryl to reduce irritation. The wound was then washed with a solution of alcohol and Hibbiscrub. Animals were allowed to recover under a warm lamp. The rats were then rehoused in solid floor cages (in small numbers) under controlled conditions of light and temperature, with free access to food and water, as before. The guidelines of the IASP were followed and the animals monitored daily to ensure good wound healing and to check whether autotomy was present.

3.1.(b) Animal preparation for microprobe experiments

Urethane (Sigma, Chemical UK.) anaesthetised rats were used in all experiments. Induction of anaesthesia was by intraperitoneal (i.p.) injection of urethane (25% weight/volume solution (w/v) 1.5g/kg) in sterile Ringers solution. The depth of anaesthesia was regularly assessed by continuous measurement of mean arterial blood pressure and ensuring that the corneal blink and hindpaw withdrawal reflexes remained absent. Further injections of urethane were given i.p. when necessary to maintain a satisfactory level of anaesthesia. Throughout surgery the rat was placed on an thermostatically controlled heat blanket linked to a rectal probe to maintain the animal's core temperature between 36-38°C.

Initial surgery involved the cannulation of an external jugular vein, to allow subsequent drug administration, and of a carotid artery to permit direct measurement of arterial blood pressure via a pressure transducer. A trachea cannula (a T-piece) was inserted to aid unobstructed breathing, with intermittent suction to ensure the trachea was not obstructed. The use of a stainless steel T-piece enabled the animal to be attached to a ventilator when artificial ventilation was necessary and permitted continuous monitoring of end tidal CO_2 levels (maintained at approximately 4%) by sampling from the side arm (stem of the T). Blood oxygenation was assisted by directing a gentle jet of humidified oxygen towards the opening of the tracheal cannula when the animal was breathing normally and towards the input of the pump during artificial ventilation.

On the right side of the rat, an incision was made at midthigh level and the sciatic nerve approached by separating the flexor muscle of the knee and freed for over 2cm proximal to its trifurcation. The animal was then placed in ventral recumbency and the skin over the dorsum was incised rostrocaudally. The connective tissue overlying the muscles of the transversospinalis muscle group was then cut longitudinally either side of the dorsal vertebral processes. Transversospinalis and

longissimus muscles were then retracted laterally from the dorsal surface of the vertebrae by blunt dissection.

Next the rat was transferred to a stereotaxic frame (Preclinical Veterinary Sciences, University of Edinburgh) and the thermostatically controlled heat blanket was replaced by a metal plate with inserted heating elements (Preclinical Veterinary Sciences, University of Edinburgh) which surrounded but did not touch the animal. This and the rectal probe were attached to a heat control box to maintain the animal's core temperature between 36-38°C. The exposed parts of the rat including the head and tail were covered with a foil blanket to reduce heat loss. The preparation was held in place by ear bars, jaw vice and spinal swan-necked clamps positioned under the vertebral transverse processes on each side of the vertebral column. Three pairs of clamps were used each pair being separated by at least one process. The middle clamp was positioned at the process corresponding to the 'floating rib' which is attached to T13 vertebra. An extended laminectomy at vertebral level T12 to L2 exposed the dura mater of the L2-L6 spinal cord segments. The dorsal surface of the spinal cord was then removed with rongeurs and the exposed spinal cord temporarily covered with cotton-wool soaked in sterile Ringer's solution.

A pool was formed by pulling up with sutures the edges of skin surrounding the incision. The cotton-wool covering the exposed spinal cord was removed and under a dissecting microscope, the edges of the laminectomy were packed with a sterile haemostatic gauze (Spongel, Houde Laboratories) to prevent subsequent bleeding. To provide further stability, a thin layer of a sterile solution of agar in Ringer's solution (4% w/v, Unipath Ltd, UK.) was poured over the entire pool. At proposed sites of antibody microprobe insertion, a rectangular area of agar was removed exposing the spinal cord. The dura mater was opened with sterile watchmakers' forceps, ensuring pressure was relieved at both sides of the cord. This pool was then continually irrigated with sterile Ringer's solution at 37°C to prevent drying of the cord surface. This continuous irrigation minimised the collection of

inflammatory exudates on the surface of the cord as excess fluid was removed by suction at an edge of the opening.

The right hindpaw was elevated and the skin overlying the midthigh incision stitched to a pool-maker to expose the sciatic nerve. This nerve was mounted (intact) on platinum stimulating electrodes and the area flooded with paraffin oil heated at 37°C to create a pool.

3.1.(c) Experiments involving stimulation of high threshold afferents of the sciatic nerve

In experiments involving stimulation of high threshold afferents animals were artificially ventilated. To maintain cord stability, neuromuscular paralysis was necessary as such stimuli produced excessive limb movements. The neuromuscular blocker, gallamine triethiodide (Flaxedil, 3mg/kg) was administered by injection into the cannulated vein. Thresholds for nerve stimulation were determined before neuromuscular blockade and the depth of anaesthesia assessed prior to each injection of gallamine. In all cases the effects of gallamine were allowed to wear off to allow verification that the level of urethane anaesthesia was satisfactory.

3.1.(d) Experiments involving spinalisation of the spinal cord

In those experiments where the spinal cord was sectioned, the spinal cord laminectomy was extended to include more of the lower thoracic cord. At the time of spinalisation, the use of another micromanipulator allowed access to both sides of the cord in the region of T9-10 where 5µl of Xylocaine 1% solution was micropipetted. The spinal cord was then teased apart using watchmaker forceps with extra care being taken to leave the dorsal vein intact by just sectioning the cord on either side of this

vein. This preparation was then left for 1 hour before microprobes were inserted into the spinal cord.

In the majority of experiments, a microprobe was filled with pontamine sky blue dye and inserted into the spinal cord to a set depth. Current was passed to eject dye as an anion from the probe tip. At the end of each experiment the animal, still anaesthetised was killed by means of intravenous injection of a concentrated solution of sodium pentobarbitone (1ml/1.5kg; Euthesate, Willows Francis Veterinary, UK) which resulted in cardiac arrest within a few seconds. The spinal cord containing the dye spot was removed, fixed in formaldehyde and subsequently sectioned to provide a direct measure of the depth of microprobe insertion (see figure 4).

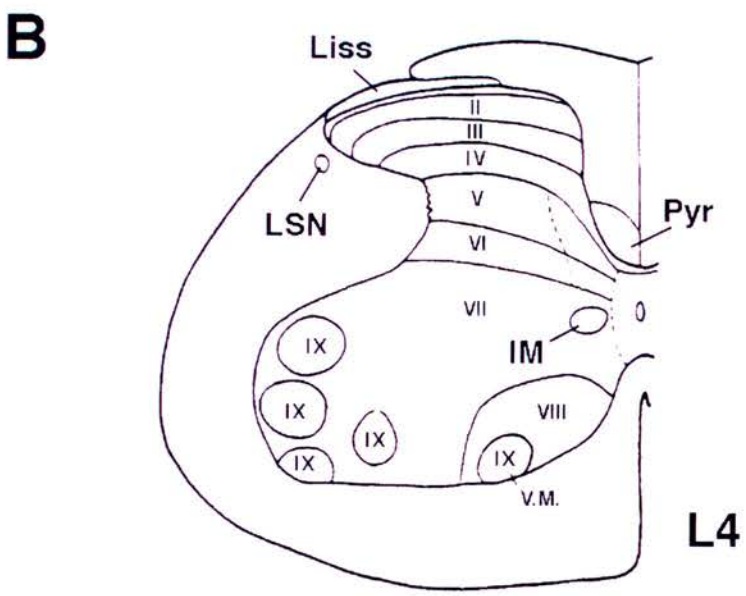
3.1.(e) Experiments involving stimulation of high threshold afferents of the sciatic nerve after nerve injury

In all experiments involving electrical stimulation the sciatic nerve was stimulated proximal to the ligature. When the high threshold afferents were stimulated the animals were artificially ventilated to maintain cord stability. The neuromuscular blocker vercuronium bromide (Norcuron 1.0mg/kg) was administered by injection into the cannulated vein. Flaxedil was not used as it has been found to increase spontaneous firing in complete nerve injury model as it has been found to block potassium channels (Michaelis et al, 1996). Thresholds for nerve stimulation were determined before neuromuscular blockade and the depth of anaesthesia assessed prior to each injection of vercuronium.

Figure 4. Histological section of the rat spinal cord.

(A) Transverse 52μm section of the lumbar region of the rat spinal cord from an antibody microprobe experiment studying the release of ir-NPY. To verify the placement of microprobes, pontamine sky blue was ejected iontophoretically at defined depths near some of the sites of prior microprobe insertion, which in this particular section was 1.1mm below the surface of the cord dorsum. The spinal cord was removed from the animal, fixed in 10% formal saline and mounted on a microtome in 0.25% agar. Transverse 52μm frozen sections were cut and stained with neutral red. (B) is a schematic drawing depicting the lamination of the spinal grey matter of the fourth lumbar spinal segment of the adult rat (derived from Molander et al, 1984). This can be seen to bear a resembalance to the spinal cord section


A
0.5 mm


B
Liss
II
III
IV
V
VI
VII
LSN
Pyr
IM
IX
IX
IX
IX
VIII
IX
V.M.
L4

In all cases the effects of vercuronium were allowed to wear off to allow verification that the level of urethane anaesthesia was satisfactory. At the end of each experiment the animal was killed as as previously described. Once dead the sciatic nerve was removed from the animal. Figure 5 shows four chromic gut ligatures surrounding a section of the sciatic nerve removed from the animal 14 days after the initial injury.

Figure 5. Sciatic nerve 14 days after ligature placement.

The ligatured nerve was found to be oedematous, especially distal to the ligatures with extensive tissue reaction around the 4 chromic gut ligatures. Some of this tissue mass has been removed so that the ligatures can be seen.


1mm

CHAPTER 4: General Methodology II: Preparation and Use of Antibody Microprobes

4.0 GENERAL METHODOLOGY II

4.1 PREPARATION OF ANTIBODY MICROPROBES

4.1.1. ANTIBODY AND PEPTIDE STORAGE

A large number of chemicals are used in the preparation and use of antibody microprobes. Most of these are inorganic and their handling and storage is straightforward. The organic compounds such as antibodies are not so robust and certain precautions were taken in their use.

4.1.1.(a) Antibodies

All antibodies for neuropeptide Y (NPY) were purchased commercially from Peninsula Laboratories Europe Ltd, and were delivered as freeze dried lyophilates. It is possible to store these lyophilates for several weeks prior to reconstitution, without any apparent loss of integrity of the antibody. These lyophilates already contain buffering salts and bovine serum albumin (BSA) resulting in the addition of a minimum volume of diluent being required to fully dissolve them. Lyophilates were reconstituted in distilled and Millipore filtered water and the resulting solution was immediately divided into 0.5ml aliquots in capped, polythene Eppendorf tubes. These aliquots were then stored at -20°C until used. As with all organic solutions, the unused portion of any thawed aliquot of antibody was discarded, as re-freezing promotes the breakdown of such compounds.

All antisera purchased were polyclonal and thus contained immunoglobulins of a ranging affinity and specificity. The absolute number of antibody molecules in a given volume of solution is not determinable when purchasing such commercial

antisera designed for use in radioimmunoassay studies. However, one batch of antisera was sufficient for 25 microprobe experiments, and the concentration of antibody used was constant in all experiments. The suitability of a new batch of antisera for use in microprobe experiments was tested by a variety of in vitro tests.

4.1.1.(b) Peptides

These were purchased in freeze-dried form and were usually diluted with a solution of phosphate buffered saline (PBS) containing 0.1% sodium azide. Peptides were reconstituted to various concentrations and stored as 100µl aliquots in capped Eppendorf tubes at -20°C. The peptide was stored as aliquots of 10^{-5}M, 10^{-6}M, 10^{-7}M and 10^{-8}M. All peptides were stored in Eppendorfs treated with Sigmacote (Sigma Chemical, UK) using pipette tips (also treated with Sigmacote) to prevent the peptide adsorbing to the plastic and thus reducing the concentration of the peptide in solution. No peptide aliquots were refrozen after being thawed.

4.1.1.(c) Radiolabelled peptides

Commercially ^{125}I-labelled NPY was purchased from Amersham being labelled with Bolton and Hunter's reagent. Each batch was purchased as a freeze dried lyophilate and was reconstituted immediately on delivery with the recommended diluent (distilled water) to a total volume of 100µl and then divided into 2.5 and 5µl aliquots. These aliquots were stored in a dedicated, lead screened freezer at -20°C until required. Following thawing, single aliquots were initially diluted in a solution of PBS/azide containing 0.5% BSA. Better results, however, were achieved with 0.5% casein instead of BSA when using NPY radioactivity (Grouzmann et al, 1992). The radioactivity was diluted on the day of use to give a final count of 2000 counts minute^{-1} µl^{-1} and was then stored on ice until required. Unused aliquots of

radioactivity were discarded at approximately 6 weeks after their specific activity date as ^{125}I-labelled peptides have a half life of 59.6 days.

4.2. PREPARATION OF ANTIBODY MICROPROBES

The technology of antibody microprobe production has been reviewed by Duggan (1991), but details of the preparation of antibody microprobes have changed in various subtle ways since then. What is presented here is the method used during the present experiments. Initially, microprobes are manufactured and coated with siloxane.

4.2.1. SILOXANE POLYMER COATING OF GLASS MICROPROBES

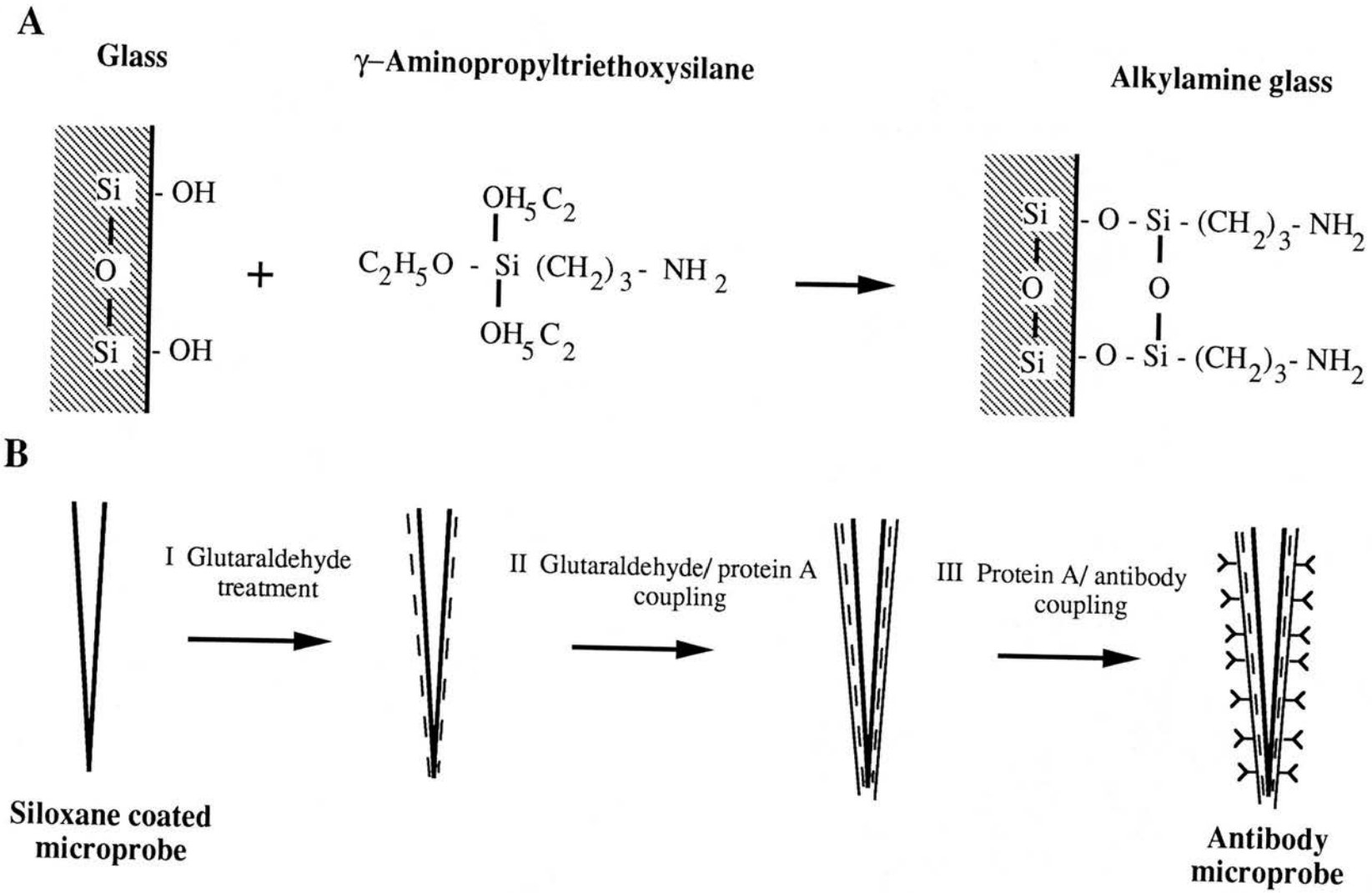
Gamma-aminopropyltriethoxysilane is a substituted silane and a member of the organosilicon class of compounds. The organic groups of this molecule can be used to bind proteins to glass. The initial reaction is shown in Figure 6. The hydroxyl groups interacting with the substituted silane are shown as part of the glass, but there is evidence that much of the reaction depends upon chemisorbed water molecules.

4.2.1.(a) Preparation of glass microprobes

Hollow, borosilicate capillary glass (outer diameter 3mm, inner diameter 1.62mm, non-filament GC 300-10, Clark Electrochemical Instruments, UK) was used to manufacture the antibody microprobes. For the initial cleaning, this glass was incubated for over 20 hours in a container of xylene followed by 3 x 30 minute washes each in a different container of absolute alcohol. The glass was then dried in a

Figure 6. Preparation of antibody microprobes

(A) shows the interaction between γ-aminopropyltriethoxysilane and the glass surface of a micropippette to produce alkylamine (siloxane coated glass). (B) shows the stages in the preparation of the antibody microprobes with the sequence of reagents used to couple antibodies to siloxane coated microprobes.


A
Glass
γ–Aminopropyltriethoxysilane
Alkylamine glass
Si
O
Si
- OH
- OH
+
OH_5C_2
C_2H_5O - Si $(CH_2)_3$ - NH_2
OH_5C_2
- O - Si - $(CH_2)_3$ - NH_2
O
- O - Si - $(CH_2)_3$ - NH_2
B
I Glutaraldehyde treatment
II Glutaraldehyde/ protein A coupling
III Protein A/ antibody coupling
Siloxane coated microprobe
Antibody microprobe

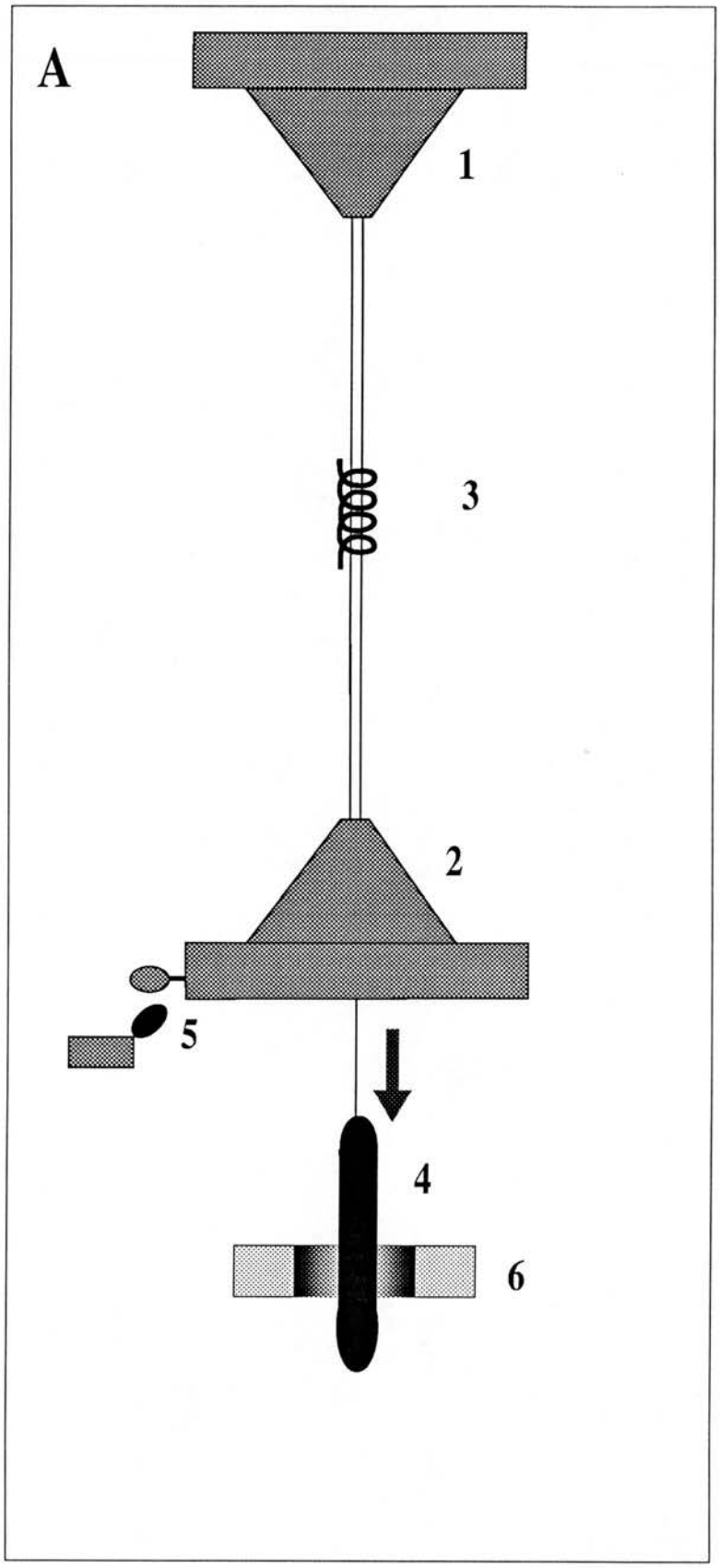
clean oven at 200°C and stored in a covered beaker until required. Each capillary was drawn out by heat and tension in a vertical microelectrode puller (Preclinical Veterinary Science, University of Edinburgh) with the settings adjusted to give an even taper from tip to shank. The platinum heating coil of the puller extended over 1cm of the glass thus ensuring the melting of a large area of glass to produce a gradual taper (see figure 7). Both ends of the microprobe were then heat sealed, the wide, unpulled end in a gas flame and the tip by touching it briefly against a molten glass bead heated by a micro-coil (Preclinical Veterinary Science, University of Edinburgh). This latter process was performed using a micromanipulator (D10 Positioner, Research Instruments UK) and a binocular microscope. The resultant glass microprobes are very flexible and thus resistant to accidental fracture during subsequent use. Microprobes were then placed in carrying glass buckets in groups of 16-20, for ease of handling. Each carrying bucket has 3 perforations in its base to permit draining during the many procedures used during microprobe production. The filled buckets were then immersed in 50% nitric acid for 30 minutes. Immersion into nitric acid promotes the formation of silanol groups on the glass surface (Weetall 1970). This was followed by 3 x10 minute washes in Millipore filtered distilled water to remove the residual acid. The buckets of probes were then dried in a clean oven at 200°C for at least 2 hours, or until further use. This process resulted in clean, dry microprobes with some degree of surface etching on which subsequent chemical reactions could proceed.

4.2.1.(b) Preparation of reagents

Clean Pyrex-glass boiling tubes (24/29, Quickfit), each large enough to hold a carrying bucket, were heated to 200°C for 2 hours in a clean oven and then placed in a rack and allowed to cool. Whilst still warm they were placed in a fume cupboard and filled with 30ml of reagent grade toluene (Aldrich, UK) followed by 10ml aliquots

Figure 7. Manufacture of glass micropipettes for antibody coating

(A) shows a schematic diagram of the microelectrode puller used. The capillary glass is suspended between two holders (1,2) running through a heated platinum coil at mid length (3). The lower holder (2) is attached to a solid metal weight (4) by means of a robust metal cable. As the glass is heated by the coil, it stretches and the lower holder drops. At a set distance a switch is activated (5) which triggers a strong electromagnetic field (6) which rapidly pulls the weight down and draws the molten glass out to a very gradual taper before separating to give two micropipettes. The heat of the coil, the distance dropped before the magnetic pull is triggered and pull force are all factors which determine microprobe shape. (B) This shows the typical dimensions of antibody microprobes used for detection of neuropeptide Y in the studies described in this thesis.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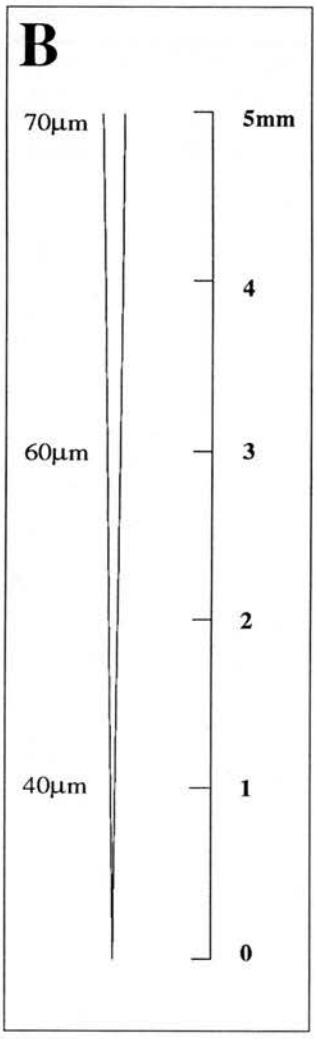
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0

of gamma-aminopropyltriethoxysilane (APTES; Aldrich) taken from a freshly opened 100ml bottle (it was usual to prepare 8-10 such tubes in each session of microprobe production, with the unused APTES being discarded). Five and a half microlitres of distilled water was added to each boiling tube which was then closed with a polythene cap. This was added in an attempt to control the amount of siloxane polymerisation at the surface of the microprobes. The toluene solution had been previously treated with molecular sieves (150g of sieves added to 2.5 litres of reagent toluene) for at least 24 hours, the purpose of the molecular sieves being to remove as much water as possible from the APTES/toluene mixture, as excessive water in the solution interferes with the even build up of siloxane polymer on the surface of the microprobes. The boiling tubes were vortexed to ensure adequate mixing of the solutions.

The buckets of glass microprobes were carefully removed from the oven with metal forceps and as quickly as possible, the boiling tubes were then opened, and the buckets of microprobes lowered into the APTES/toluene mixture. Each tube of reagent, now containing a bucket of microprobes was then restoppered and removed from the fume cabinet. Boiling tubes were then placed in a centrifuge (Mistral 2L, MSE Science Instruments, UK) and spun at 6°C, 2000rpm for 1 hour. This centrifugation resulted in a more even build up of siloxane polymer on the surface of the microprobes. This may be due to the centrifugation of free water away from the tips of the microprobes or to the removal of small particle of debris from the solution around the tips which may act as foci for the polymerisation reaction. The stoppered tubes were then removed from the centrifuge and left at room temperature for at least 20 hours. The buckets of microprobes were the removed from the boiling tubes and the APTES/toluene mixture was discarded. The buckets of microprobes were briefly washed by dipping in toluene solution and then allowed to dry at room temperature. One microprobe from each bucket was inspected under a binocular microscope at x125 magnification to ensure that the siloxane coating was visible: if too light the

procedure was repeated. The buckets of microprobes were then placed in an oven at 200°C for at least 24 hours to cure the siloxane polymer. This heating cures the polymer to a stable water-insoluble form and maximises the number of amine groups free from steric hindrance (Chiang et al, 1982).

4.2.1(c) Assessment of siloxane coating

Scanning electron micrographs have been taken of the surface of uncoated and siloxane coated microprobes. These have indicated that the polymer coating not only provides a chemical base for the subsequent immobilisation of antibodies to the microprobe but also vastly increases its surface area. (Duggan et al, 1988a).

A good polymer coating on the surface of the microprobe will appear, under x 40 magnification using incident light, as a milky deposit on the surface of the microprobes; white prior to heat curing and yellowish afterwards. The microprobes are subsequently used to detect peptide binding with a resolution of 100μm and hence assessment of the eveness of polymer coating in this way is not sufficiently accurate. Therefore, following curing, individual microprobes were placed in a micromanipulator mounted under a binocular transmission light microscope and examined at x 125 magnification to examine the siloxane coating accurately.

This technique produces microprobes with a fine granular siloxane coating. It was important to identify microprobes with uneven or patchy distribution of siloxane coating, which were discarded, and also to identify microprobes with small focal aggregations of polymer. In some cases these 'lumpy' microprobes could be made usable by wiping the tip vigorously with soft tissue. This tended to remove irregular lumps of siloxane polymer whilst leaving the underlying layers intact. Only microprobes with a very even coating of siloxane polymer passed this selection stage as suitable for *in vivo* experiments. Microprobes were sorted into three groups of coating; light, medium and heavy and were stored horizontally in specially prepared

grooved metal racks inside covered boxes, until required. The covered boxes protected the probes and ensured they were kept free from dust. Although the analysis of microprobe images is performed in such a way as to minimise the effects of coat density on the final result, for each experiment microprobes with similar densities of siloxane were used to minimise the differences in antibody coating.

4.2.2. THE PREPARATION OF ANTIBODY MICROPROBES FOLLOWING SILOXANE COATING

4.2.2.(a) Antibody coupling to microprobes

The binding of antibodies to siloxane coated microprobes takes a minimum time of 48 hours therefore the preparation of antibody microprobes must be timed in relation to proposed experiments to minimise wastage. Siloxane coated microprobes can be stored for prolonged periods, but once organic molecules have been linked to the microprobe coating they have a relatively short lifespan. There are three stages in the coupling of antibodies to the siloxane microprobes, each stage being designed to maximise both the specifity and affinity of the finished microprobes for the peptide of interest. Glass capillaries were used as a convient way of incubating the microprobes in minimal quantities of protein, peptides and radiolabelled peptide since these are all expensive.

Protein A (Sigma Chemical, UK) a component of the cell wall of Staphylococcus aureus was bound onto the surface of the polymer to adsorb selectively the IgG fraction of the antisera. If a monoclonal antibody concentrate virtually free of unwanted proteins was available, then this antibody could be coupled directly to the siloxane coating and incubation in protein A would not be necessary. However, in this study the antisera were raised from rabbit immunisation and treatment with protein A was necessary to immobilise the desired immunoglobulin as the antisera were polyclonal and hence contained immunglobulins of a ranging affinity and specificity. This protein binds to the Fc portion of immunoglobulin of the IgG class, raised in rabbits and guinea-pigs, whilst leaving the peptide binding site free (Goding 1978).

(ai) Glutaraldehyde treatment

The bifunctional reagent glutaraldehyde was used as a cross-linking molecule to react covalently with the free amine groups of the siloxane polymer and amine groups of protein A. Siloxane coated probes were selected (and their coatings given a final check under the microscope) in the desired numbers for the proposed experiments. Microprobes were placed in groups of 12 in clean carrying buckets (not those used in siloxane preparation) and immersed in a 2.5% solution of glutaraldehyde (BDH Laboratory Suppliers, UK) in distilled water for 30 minutes. The buckets of microprobes were then washed for 3 x 10 minutes in distilled water before proceeding immediately to step 2.

(aii) Glutaraldehyde / Protein A coupling

Protein A was diluted in phosphate buffered silane (PBS) containing 0.1% sodium azide, to a final concentration of 0.1mg ml^{-1}. Five microlitre glass capillaries (Blaubard, UK) were filled with protein A by capillary action. Under a dissecting microscope, the tip of each glutaraldehyde treated microprobe was inserted into one of the protein A filled capillaries with care taken not to touch the capillary internal wall with the tip as it entered the capillary. If the sealed microprobe tip appeared to be broken at this stage then the microprobe was discarded. Each microprobe with its tip inside a capillary was then placed horizontally on a perspex rack. The rack was placed in a covered plastic tray containing on its base tissue paper soaked in PBS/azide. This fluid was to prevent loss of the protein A solution from the capillaries by evaporation during the subsequent incubation. The covered racks were then placed in a cold room at 6°C for at least 24 hours.

(aiii) Protein A-Antibody coupling

Following incubation in protein A, microprobes were removed from their capillaries and placed in glass tanks containing sodium borohydride (Sigma Chemical, UK.) 2.5%w/v in borate buffer for 5 minutes. During this time, the microprobes were repeatedly lifted out of solution to disperse the hydrogen bubbles from their surface. The ensuing reaction, which produces free hydrogen, reduces the Schiff bases which form by aldehyde coupling to amino groups on the protein A. The microprobes were then replaced in the handling buckets and given 3 x 10 minute washes in PBS/azide solution. Glass capillaries (20µl, Camlab, UK) were filled with the required antibody solution and the tip of each microprobe was inserted into a capillary as previously described. The microprobes were then placed on perspex racks in covered boxes and incubated at 6°C for at least 24 hours.

4.3. *IN VITRO* TESTING OF ANTIBODY MICROPROBES

Before use *in vivo*, antibody microprobes were subjected too numerous *in vitro* tests of their selectivity and affinity for the ligand being studied. This *in vitro* testing was carried out in two stages: firstly, by extensive *in vitro* checking of antibody microprobes prepared with a newly purchased batch of antibody, prior to planning any in vivo work with the antibody and secondly, an extensive check of microprobe function concurrent with *in vivo* experiments.

When using a new antisera attention was paid to the manufacturers claims regarding the cross-reactivity of the antisera with other peptides. Thus, for the C-terminally directed antibody to neuropeptide Y used throughout these studies, Peninsula Laboratories stated that the antibody cross-reacted with peptide YY a member of the pancreatic peptide family due to its close sequence homology to

neuropeptide Y, but had negligible cross reactivity with non members of this family such as VIP, amylin, insulin, galanin and somatostatin. This antibody was stated as having 100% cross-reactivity for human, rat and porcine full length sequences of neuropeptide Y, but only 3% cross-reactivity with pancreatic polypeptide and negligible cross-reactivity with Prepro NPY 68-97. It was important to verify the manufacturers claims for antisera specificity using the antibody microprobes prior to commencing *in vivo* work.

4.3.(a) Newly purchased antibody

A number of antibody microprobes were prepared as previously described. These were divided into groups of approximately five and marked with indelible pen to allow their subsequent identification. The microprobes were placed in carrying glass buckets and washed for 15 minutes in PBS/azide solution. These microprobes were then inserted into 5µl capillaries filled with various peptide solutions, and incubated for 30 minutes at 37°C in a humidified box (to prevent evaporation of the peptide solutions from the capillaries) and to simulate *in vivo* conditions. The capillaries were treated with Sigmacote to prevent peptides sticking to the glass wall and thus required filling under pressure, as the solution would no longer rise by capillary action. The peptide solutions used might typically be as follows and as shown in table 3:

Group 1- 0M, no incubation in peptide

Group 2- 10^{-5} M NPY

Group 3- 10^{-6} M NPY

Group 4- 10^{-7} M NPY

Following their incubation in peptide the microprobes were washed in the presence of a magnetic stirrer in a chilled solution of PBS containing 0.1% Tween 20. The microprobes were left in the solution, tips down, for 15 minutes with the aim of

removing unbound peptide from their surfaces. After washing, the tips of the microprobes were placed in 5µl capillaries filled with a solution of Bolton-Hunter labelled ^{125}I-NPY, diluted in PBS/azide containing 0.5% casein diluted to give 2000 counts minute^{-1}ul $^{-1}$. Casein was present in the radioactivity solution to block non-specific radiolabelled peptide binding to the microprobe surface (Grouzmann et al, 1992). The microprobes were then incubated in the capillaries filled with radioactivity solution for 24 hours at 6°C.

The probes were again washed for 15 minutes in chilled PBS/Tween 20. This time, however, the thick ends of the microprobes were mounted in a sealed perspex block which in turn connected to a vacuum pump. Probes were washed under suction at this stage to suck the washing solution through the inside of the microprobes, thus removing any radiolabelled peptide that might have been drawn in during the preceding incubation.

Next, the terminal 10mm of the microprobes were broken off, between a thumb and forefinger and were then fixed to narrow strips of cartridge paper with a small amount of white liquid typing paper applied to the thick end. These strips of card were numbered according to the number written on the probe shank at the start of the procedure, to allow subsequent identification. Each strip of card was placed in a separate gamma-counter tube, and the amount of radioactivity, in counts minute $^{-1}$ was determined for each tube using a gamma. Lastly, the strips of card bearing the microprobe tips were fixed to a sheet of cartridge paper. This sheet of paper was placed in a standard (non-screen) x-ray film cassette with a sheet of monoemulsion x-ray film (CEA, Sweden) and left for several days to produce autoradiographic images of the microprobes thus ensuring that a permanent record of the *in vitro* testing was obtained.

4.3.(b) Autoradiographs

With *in vitro* testing, the terminal lengths of the antibody microprobes are evenly exposed to radioactivity, so the autoradiographs should show an even distribution of tracer binding. Study of these autoradiographs allows a very sensitive assessment of the evenness of the distribution of antibody on the microprobe surface. The siloxane polymer can itself be visualised under the microscope, but the examination of autoradiographs is the only way to monitor the even application of the organic layers to the antibody microprobes. Where *in vitro* microprobes produced poor quality images, the procedure was repeated and if this re-occurred then it was attributed to a degeneration of the radiolabelled peptide solution which was replaced. On the rare occasions where the autoradiographic images of an *in vitro* antibody had lumps, the radioactive count obtained from such probes were discarded and further microprobes with similar coating were labelled with $F(ab)_2$ fragments of an antibody raised against the Fc region of IgG of the species in which the original antiserum was raised. These $F(ab)_2$ treated microprobes were processed in a similar manner as before to obtain autoradiographs. These almost invariably resulted in even coats, indicating that the antibody coating was indeed even.

4.3.(c) *In vitro* testing -measurement of microprobes sensitivity and selectivity

In table 3, microprobes in group 1 are the control microprobes that have not been incubated in unlabelled peptide and thus represent zero NPY. The amount of bound radioactivity to these microprobes is dependent on the actual amount of antibody bound to the probe surface and the binding affinity of the particular antibody used which may vary from batch to batch. The antibody used was not changed during a series of experiments. Groups 2, 3 & 4 give an indication of the sensitivity of the microprobe assay and its suitability for *in vivo* application. Incubation in peptide for which the antibody shows affinity, reduces the number of antibody binding sites

Table 3-The results of an in vitro assay testing the sensitivity of microprobes bearing antibodies to NPY

Microprobe tips were mounted on card and placed in tubes and assessed for amount of radioactive emission (in cpm) using a gamma counter. The mean ± SEM were obtained for microprobes that had been incubated in the same conditions, using cpm values (or 'counts') that had been corrected for background emissions (cor.cpm).

Group 1: microprobes incubated at 0M were not exposed to any concentration of peptide solution but simply incubated in [^{125}I]-NPY. The mean ±SEM of the cor.cpm obtained for these '0M' microprobes represent 100% binding of the radiolabelled peptide.

Group 2: microprobes incubated at 10^{-5}M NPY resulted in such small counts that these were not measurable by the gamma counter. Suppression of the mean counts obtained with 0M (i.e. the total counts) was 100%.

Group 3: microprobes incubated at 10^{-6}M NPY resulted in an overall suppression of the total counts by 83.0%

Group 4: microprobes incubated at 10^{-7}M NPY resulted in an overall suppression of the total counts by 51.2%

IN VITRO RESULTS-TABLE 3

Group no.	Concentration of peptide incubation	Cor.cpm	Mean cpm ± SEM	% reduction of mean
1	OM	676.5		
1	OM	586.9		
1	OM	554.5	614.9 ± 30.9	----
1	OM	555.5		
1	OM	700.9		
2	10^{-5}M NPY	0		
2	10^{-5}M NPY	0		
2	10^{-5}M NPY	0	0	100%
2	10^{-5}M NPY	0		
2	10^{-5}M NPY	0		
3	10^{-6}M NPY	154.4		
3	10^{-6}M NPY	90.8		
3	10^{-6}M NPY	86.8	104.5 ± 12.6	83.0
3	10^{-6}M NPY	97.8		
3	10^{-6}M NPY	92.8		
4	10^{-7}M NPY	319.3		
4	10^{-7}M NPY	127.9	300.2 ± 60.4	51.2
4	10^{-7}M NPY	408.7		
4	10^{-7}M NPY	344.7		

available for the subsequent radioligand binding. Thus the amount of radioactivity that binds to the microprobes is reduced compared to the values obtained for control microprobes. In this assay radiolabelled binding was found to be completely suppressed by 10^{-5}M of NPY. However, comparing all *in vitro* tests, the average suppression by 10^{-5}M of NPY has been found to be 80%. In this assay, the radiolabelled binding was suppressed by 83% with 10^{-6}M of NPY and by 51.2% with 10^{-7}M of this peptide.

Previous microprobe studies on tachykinin release in the dorsal horn of the spinal cord have shown that where radiolabelled tracer binding was suppressed by 50% by prior incubation in a 10^{-7}M solution of unlabelled ligand, microprobes were found to be effective in detecting release *in vivo*. In all studies presented here for NPY, the suppression of tracer binding by 10^{-7}M ligand was in this range, but prior to actually performing *in vivo* experiments it was unknown if this would be adequate to detect endgenous NPY.

The in vitro result shown in table 4 gives an indication of the selectivity of the NPY antibody when present in the spinal cord. Galanin and VIP have been chosen, as unlike pancreatic polypeptide, they are present in the spinal cord and are both found to increase after peripheral nerve injury. Minimal reduction in the radioactivity counts should occur when microprobes are incubated in a structurally non-related peptide. The radiolabelled binding was suppressed by only 3% with 10^{-6}M of galanin and by 2% with 10^{-6}M of VIP. This result is comparable to the manufacturers claim that the NPY antisera shows no cross-reactivity for VIP or galanin. This was the case with all antisera used in these studies.

The *in vitro* assay result shown in table 5 indicates the ability of this peptide to recognise PYY, a closely related member of the pancreatic family. The radiolabelled binding was suppressed by 57.5% with 10^{-5}M of PYY and by 40.9% with 10^{-6}M and 37.5% with 10^{-7}M. This result agrees with the manufacturers claim

Table 4-The results of a typical *in vitro* assay testing the specificity of microprobes bearing antibodies to NPY.

Group 1-microprobes incubated at 0M were not exposed to any concentration of peptide solution but simply incubated in [^{125}I]-NPY. The mean ±SEM of the cor.cpm obtained for these 0M microprobes represent 100% binding of the radiolabelled peptide.

Group 2-microprobes incubated at 10^{-6}M of galanin, a peptide unrelated to the pancreatic polypeptide family, resulted in an overall suppression of the mean counts obtained with 0M (i.e. the total counts) by only 3%.

Group 3-microprobes incubated at 10^{-6}M of VIP, a peptide unrelated to the pancreatic polypeptide family, resulted in an overall suppression of the mean counts obtained with 0M (i.e. the total counts) by only 2%.

TABLE 4

Group no.	Concentration of peptide incubation	Cor.cpm	Mean cpm ± SEM	% reduction of mean
1	0M	671.3		
1	0M	488.70		
1	0M	667.7	498.6 ±75.7	
1	0M	671.3		
1	0M	357.2		
2	10^{-6}M galanin	378.9		
2	10^{-6}M galanin	590.7	483.5 ±43.2	3.0
2	10^{-6}M galanin	479.4		
2	10^{-6}M galanin	485.1		
3	10^{-6}M VIP	581.6		
3	10^{-6}M VIP	385.9	488.6 ± 62.4	2.0
3	10^{-6}M VIP	610.7		
3	10^{-6}M VIP	376.2		

that the NPY antisera cross-reacts with PYY. The degree of suppression obtained in this assay was less than the suppression obtained with NPY in table 4.

4.3.(d) *In vitro* tests concurrent with *in vivo* experiments

Periodically, during *in vivo* experiments, microprobes were chosen with a coat similar to that used *in vivo* to carry out small *in vitro* tests to ensure the assay was satisfactory. This test was performed to confirm that the microprobe affinities and sensitivities were still as expected from the earlier *in vitro* investigations. They were usually divided into three groups, no incubation in peptide and incubation with two concentrations of NPY unlabelled peptides to confirm that suppression was occurring.

4.4. *IN VIVO* USE OF ANTIBODY MICROPROBES

Prior to the insertion of antibody microprobes into the spinal cord, the suitability of potential penetration sites was examined. The spinal cord irrigation was temporarily stopped and the sterile Ringer's solution removed from the cord surface by gentle suction at an edge of the agar pool. A silver ball electrode was then gently pressed on to the dorsum of the spinal cord just medial to the line of entry of the dorsal roots and the threshold stimulus current and voltage needed to evoke a cord dorsum potential was measured. Cord dorsum potentials elicited by low threshold electrical stimulation of the sciatic nerve were displayed on a cathode ray oscilloscope. The ball electrode was positioned at several rostro-caudal locations to determine the distribution of afferent input from the sciatic nerve and hence to select sites of insertion for microprobes. The site producing the largest field potential was chosen for microprobe insertion.

Table 5-The results of a typical in vitro assay testing the specificity and sensitivity of microprobes bearing antibodies to NPY

Group 1-microprobes incubated at 0M were not exposed to any concentration of peptide solution but simply incubated in [^{125}I]-NPY. The mean ±SEM of the cor.cpm obtained for these 0M microprobes represent 100% binding of the radiolabelled peptide.

Group 2-microprobes incubated at 10^{-5}M of peptide YY (PYY) a closely-related member of the pancreatic polypeptide family, resulted in an overall suppression of the mean counts obtained with 0M (i.e. the total counts) by 59.9%.

Group 3-microprobes incubated at 10^{-6}M of PYY resulted in an overall suppression of the total counts by 40.9%.

Group 4-microprobes incubated at 10^{-7}M of PYY resulted in an overall suppression of the total counts by 37.5%.

TABLE 5

Group no.	Concentration of peptide incubation	Cor.cpm	Mean cpm ± SEM	% reduction of mean
1	0M	554.4		
1	0M	579.9		
1	0M	668.2	573.4 ± 32.6	----
1	0M	597.2		
1	0M	467.2		
2	10^{-5}M PYY	281.3		
2	10^{-5}M PYY	139.3		
2	10^{-5}M PYY	335.5	243.6 ± 41.9	57.5
2	10^{-5}M PYY	221.2		
2	10^{-5}M PYY	278.2		
3	10^{-6}M PYY	403.9		
3	10^{-6}M PYY	330.6	338.7 ±24.6	40.9
3	10^{-6}M PYY	284.3		
3	10^{-6}M PYY	336.1		
4	10^{-7}M PYY	409.6		
4	10^{-7}M PYY	310.1		
4	10^{-7}M PYY	296.8	358.3 ± 22.9	37.5
4	10^{-7}M PYY	389.5		
4	10^{-7}M PYY	385.5		

Microprobes, prepared as described earlier, were removed from the 20µl capillaries containing antisera to neuropeptide Y(NPY) and placed in carrying buckets. The microprobes were washed in PBS/azide solution at room temperature for 15 minutes prior to use, to wash away any excess antibody i.e antibody which had not bound to the microprobe surface. Both sealed ends were removed, the thick end first using a glass saw to scratch the surface of the glass which was then broken off between thumb and forefinger. This must be done carefully or the tip of the microprobe will shatter. Next, the tip was broken back to a diameter of approximately 10µm by gently bumping it against the surface of a metal coil under a binocular microscope at x 125 magnification. Again the adequacy of the siloxane coating was checked and any probe with an uneven coat or tip > 10µm was discarded. Microprobes were numbered using a coded system of coloured rings.

One microprobe was then placed in each of two stepping motor micromanipulators, one oriented vertical and the other 10° from vertical. The manipulators were then moved down by hand until they were close to the spinal cord surface. Spinal cord irrigation was temporarily stopped and excess Ringer on the surface of the cord removed by suction. Under x15 magnification, the tips of the two microprobes were moved in the horizontal plane and were positioned as close together as possible at the proposed site of insertion. The microprobes were then lowered with the micromanipulators until the tips were just touching the surface of the spinal cord. The micromanipulators were zeroed and then used to insert the microprobes into the dorsal spinal cord to a depth of 2.25mm from the dorsal surface.

Once the microprobes were in situ the Ringer irrigation system was turned on again. All NPY microprobes remained in the spinal cord for 15 minutes. The peripheral stimuli applied during that time, however, varied according to the experimental protocol which will be outlined separately in the Methods section for each study. Following exposure to the spinal cord, microprobes were treated in the same way as that earlier described for *in vitro* tests. The microprobes were washed

for 15 minutes in ice cold PBS/Tween 20 to remove any tissue debris that may have got trapped inside the probe and then inserted into 5µl capillaries containing 2000cpm/µl, of the radiolabelled tracer and stored at 6°C for 24 hours. The thick ends of the microprobes were mounted in a sealed perspex block which in turn connected to a vacuum pump allowed the microprobes to be washed in PBS/Tween 20 under suction to remove any labelled peptide that may have entered the lumen of the microprobe during the 24 hour period. This washing procedure also minimised non-specific binding of the radiolabelled peptide. The terminal 1cm of each microprobe was removed from the glass shaft (this was possible without shattering the tip due to the microprobes flexible shape) and was stuck directly onto a sheet of cartridge paper with white typing correction fluid, lining up in sequence *in vivo* and *in vitro* probes. The previously assigned microprobe number corresponding to each tip was written on the paper just above it. This sheet was then placed in a cassette with momoemulsion x-ray film (CEA, Sweden), the emulsion side of the film remaining in contact with the microprobes. The exposure time required to produce autoradiographs of microprobes suitable for analysis varied according to the peptide being studied. The exposure time chosen for NPY microprobes was between 7-12 days and it was usual to obtain two films with different exposure times for each experiment.

4.5. ANALYSIS OF ANTIBODY MICROPROBE AUTORADIOGRAPHS

The principles employed in the analysis of microprobe autoradiographic images were based on those described by Hendry et al (1988). Microprobe images were analysed with a computerised image analysis system utilising an Imaging Technology PC Vision plus frame grabber board operating in a Data Control Systems 286e (AT based) computer.

A satisfactory x-ray image was one with images well above background silver grain density but below the maximal density for the film. Following examination of the initial x-ray film, it was usual to expose a second film to the microprobes for a different time from that used for the first exposure to obtain a back-up copy. However, occasionally, single antibody microprobe images would be blurred due to poor apposition of the microprobe to the film and in this case a minor repositioning of the affected microprobe was made prior to repeated exposure. Double images occurred rarely due to movement of the film during storage, thus exposure to a second film was necessary. The film selected for analysis was then labelled with indelible marker pen, each image being identified by the assigned number to that microprobe prior to its use in vivo. The film was cut up into small sections, each section containing one or two complete microprobe images. Surface dust and grease were removed from each piece of x-ray film by wiping with lens tissue.

Single microprobe x-ray film images were placed on a mechanical microscope stage in a light proof box, and illuminated from beneath through a narrow slit only just wider than the microprobe image itself. The light source (2 X 25W microscope halogen bulbs) was mounted in a light box fitted with diffusion plates to give uniform distribution of light. The light source was powered by a stabilised DC power supply (Farnell) to assist stability of illumination. A video image of the autoradiograph was obtained by scanning the probe image using a Panasonic camera (a charge-coupled-device camera) and the field of view restricted to the distal 5mm of

the microprobe image The automatic gain control function of the camera was inactivated. This was essential to enable use of the video camera as a densitometer as it ensures that the digitised video image is linear with respect to the autoradiograph. This charged coupled digitised video image is linear with respect to the autoradiograph density. This charged coupled device camera produced a 512 x 512 pixel 'map' of the field of view. A memory map of the 512 x 512 locations was stored in the image board and as the length of the probe was 5000μm each location corresponded to approximately 10 x 10μm. The optical density board converted the optical density of the video image to an arbitrary greyscale value over a range of 0 to 255, where 0 is blackness and 255 is maximum light. Since the background was bright and the microprobe image was dark, the image processor utilised an "inverse look-up table". Thus a true value of 255 was converted to 0 and 0 was converted to 255 and intermediate values adjusted proportionately. This has the effect of the microscope image appearing bright against a dark background. If the light intensity is increased over 255 no further change in the greyscale reading is obtained and linearity is lost. Therefore, the light intensity was adjusted to give a value of zero when no film was present. The video image of each microprobe autoradiograph was displayed on a colour monitor connected to the output of the image processing board that allowed a constant monitoring of the illumination of the film. The amount of light reaching the camera was kept constant from one x-ray film to the next by means of varying the current passing to the light source.

Two background scans were obtained first by digitising the image from an unexposed (no microprobe image) part of the film. Once the settings have been set for any one film, they were left unchanged while all the data on that film was obtained. Next the image of the microprobe was aligned on the video monitor such that it was horizontal on the screen and its tip (which is the reference point for averaging purposes) just contacted the edge of the screen. A background subtraction was then carried out by the computer whereby the value of each pixel in the microprobe scan

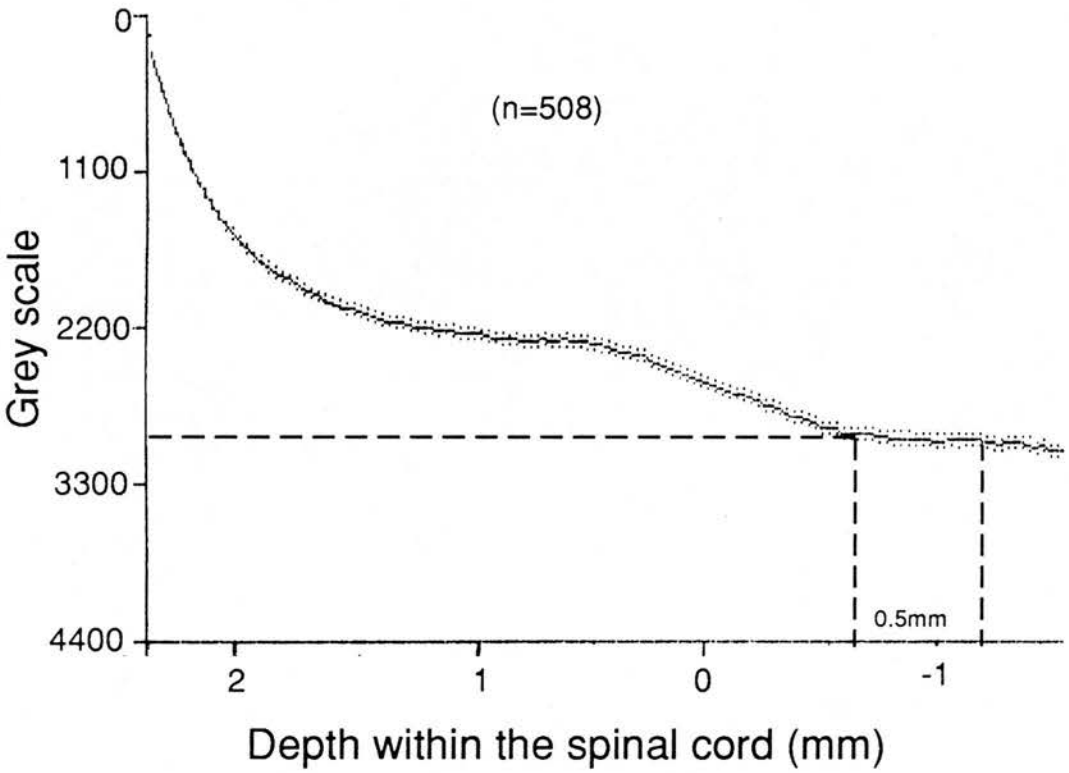
had subtracted from it the background value at that position. The program then performed transverse integrations for each vertical column of pixels across the restricted part of the field which contained the microprobe image. The corrected image was then displayed on a colour monitor in 16 false colours corresponding to 16 grey scale values. The integrals for each scanned microprobe were stored on hard disk (512 numbers to represent the 5mm of microprobe image) together with 37 coded values representing various details corresponding to the experimental parameters pertaining to that image. For each microprobe scan a plot of darkness of the image, 'greyscale' against distance along the length of the probe tip was displayed.

A small proportion of microprobe x-ray film images were found to be unsuitable for further analysis despite all the checks of microprobe quality performed during siloxane polymer coating. Such images most often showed focal intensities of radioligand binding that could not be attributed to any physiological event and likely represented dust on the probe. The antibody code of such a microprobe was altered to 99, as the computer system reads this code first and if the value is 99 the probe is discarded.

To minimise any differences due to variability in x-ray film exposure time the values obtained for each microprobe scan were 'normalised'. First the mean image analysis plot of all microprobes in a complete study is plotted, from this the mean grey value of a relatively constant 500μm section located outwith the depth of microprobe insertion into the nervous system was noted (see figure 8). The integral values obtained for the image scan were then adjusted according to the variability constant for that microprobe and entered into a new file called the 'normalised' file. If we were unable to normalise then *in vitro* microprobes would need to be included in each experiment for counting. The exposure time of the film would then be adjusted based on this radioactivity count to give comparable autoradiographic image density between experiments.

Figure 8. Determination of standard grey scale value to 'normalise' the values obtained for microprobe image scans in the NPY study

This is an example of the type of mean image density scan of NPY microprobes used to decide the standard grey scale of a relatively constant 500μm section of the grouped microprobes located in a region distal to that inserted into the spinal cord. The 500μm section region chosen is marked which approximates to a grey scale value of 3000. This was used to standardise the values obtained for each microprobe image scan in the neuropathic study. Such a process 'normalises' the values to minimise any differences between microprobes due to variability in physical factors such as X-ray exposure times.


0
1100
2200
3300
4400
Grey scale
(n=508)
0.5mm
2
1
0
-1
Depth within the spinal cord (mm)

Subsequently a data sorting program was used to group together microprobe images fulfilling stated experimental criteria. For mathematical analysis of microprobe images, the integral mean grey scale values were pooled into groups of 3, reducing the spatial resolution of the technique from 10μm to 30μm. Mean image analyses were produced for each group, by summing the greyscale values corresponding to each 30μm for all microprobes selected and plotting for each interval the mean greyscale value and the standard error of the mean. As the depth of microprobe insertion was known this was converted to a plot of greyscale against the depth of insertion in the spinal cord (in mm). The average greyscale values for a particular group of microprobes were plotted as a solid line graph with the values of the standard error of the mean plotted on one side only. Pairs of mean image analyses, representing different experimental manipulations, were compared statistically by subtracting at each 30μm point the values obtained for the control group from the experimental group to obtain a difference. This enabled a plot of the Student's t-test derived from the differences between the mean image analyses at 30μm intervals to be constructed.

CHAPTER 5: Studies of the Spinal Release of neuropeptide Y in normal rats

5.0 INTRODUCTION

Histochemical studies first suggested that NPY has a role in the spinal processing of information conveyed by primary afferents for although there is negligible NPY in primary afferents of the rat there is a dense plexus of NPY-containing terminals and fibres in laminae I & II of the dorsal horn (see section 1.2.5.).

There are, however, no reports of the release and the processes controlling release of NPY in the dorsal horn. When I commenced my studies there was a need to determine whether NPY is tonically released in both normal and neuropathic rats and if such a release is increased or decreased by peripheral nerve stimulation. The aim of this study was to employ microprobes bearing immobilised antibodies to NPY to determine if there was a basal presence of this peptide in the spinal cord of normal rats and if so, to determine it's origin. The effects of spinal transection on the spinal release of ir-NPY was also studied in the rat due to the known presence of NPY in the spinal termination of fibres of supraspinal origin (Blessing et al, 1987; Holets et al, 1988). Although the near absence of ir-NPY in dorsal root ganglion neurones implies that this compound cannot be released in significant amounts from the spinal terminations of primary afferents, peripheral inputs could still evoke intraspinal release of ir-NPY by activating spinal or supraspinal processes. This was investigated by stimulating the sciatic nerve at 3 different frequencies.

5.1 MATERIALS AND METHODS

5.1.1. MICROPROBE PREPARATION

Antibody microprobes were prepared as previously described, using a polyclonal antiserum (Peninsula Laboratories Europe Ltd) that had been raised in rabbits against the C terminal end of neuropeptide Y. Data supplied from Peninsula Laboratories indicated that this antiserum recognised porcine, human and rat full length sequences equally well as these forms differ by only 1 amino acid located at position 17. The antiserum recognised peptide YY but not prepro NPY(68-97) or pancreatic polypeptide.

5.1.2. EXPERIMENTAL PREPARATION

A total of 25 male Wistar rats (weight range 250 to 350g; Charles River Ltd, UK) were used in this study. Anaesthesia was induced and the animal prepared as previously described in section 3.1.(b). All microprobes were inserted into the spinal cord (2 at a time) with a pair of stepping motor micromanipulators to a depth of 2.25mm from the dorsal surface and left *in situ* for 15 minutes. After removal from the spinal cord, all microprobes were washed and then incubated for approximately 24 hours in NPY radiolabelled with Bolton-Hunters reagent.

In the absence of peripheral stimulation, microprobes were inserted into both sides of the cord at a level determined by the electrophysiological recordings corresponding to the site of termination of the sciatic nerve. Only one stimulation period of 15 minutes (either 1Hz, 20Hz, both 3 x threshold or 2Hz,>100 x threshold) was used per experiment. In such experiments microprobes were positioned only in

the spinal cord ipsilateral to the nerve being stimulated. Following electrical stimulation, it was usual to insert microprobes into this side of the cord for three periods of no stimulation.

In the spinalisation experiments, 2 pairs of microprobes were inserted into both sides of the cord prior to cord transection. One hour later, this protocol was repeated with a further 4 pairs of microprobes thus ensuring that both sides of the cord were sampled equally. No peripheral stimulation was used in these experiments.

5.2. RESULTS

A total of 273 microprobes coated with antibodies to neuropeptide Y were inserted into the rat spinal cord form the basis of this analysis. One hundred and thirteen *in vitro* microprobes were prepared concurrently with those used *in vivo*. An additional 440 microprobes were used for *in vitro* sensitivity tests.

5.2.(a) *In vitro* tests

Prior to use and throughout use *in vivo*, the sensitivity of the prepared antibody microprobes were tested (as outlined in section 4.3.). *In vitro* tests indicated that a 10^{-8}M solution of NPY suppressed such binding on average by 40%, with a 10^{-5}M solution resulting in over 80% suppression. Hence, it can be assumed that the non-specific binding for these microprobes accounted for less than 20% of the total binding. Information supplied by Peninsula Laboratories on the specificity of the antiserum was confirmed on microprobes by demonstrating minimum suppression of binding of $[I^{125}]$-NPY by a range of concentrations of the peptides.

5.2.(b) Preliminary experiments

In preliminary experiments, microprobes were inserted into the spinal cord for 15 & 30 minute periods in the absence of any active peripheral stimuli. The mean image analyses obtained from the two groups were compared to determine the relation between detection of extracellular immunoreactive (ir) neuropeptide Y (NPY) and the amount of time the microprobes were present in the spinal cord. A comparison was made of the mean image analysis of 38 microprobes remaining in the spinal cord for 15 minutes with the mean image analysis of 28 microprobes remaining in the spinal cord for 30 minutes. The plot of microprobes inserted into the spinal cord for 30 minutes was displayed above that of the mean image analysis of microprobes present in the spinal cord for 15 minutes over a restricted range (not illustrated). However, these differences were found to be significant only at a few 30 μm intervals at approximately 1.0-1.2mm from the dorsal cord surface. As the differences in the detection of ir-NPY using two time periods were minimal, all further studies thus used microprobes present in the spinal cord for 15 minutes since more results could be obtained per experiment.

5.2.(c) Basal presence of ir-NPY in normal rats

The primary aim of this study was to investigate the release of ir-NPY in animals with a peripheral nerve injury. It was therefore first necessary to investigate the release of ir-NPY in normal animals to allow comparisons to be made. Thus it was essential to determine in normal rats:

1/ if there was a basal presence of ir-NPY.

2/ if basal release was present to determine the source of such release.

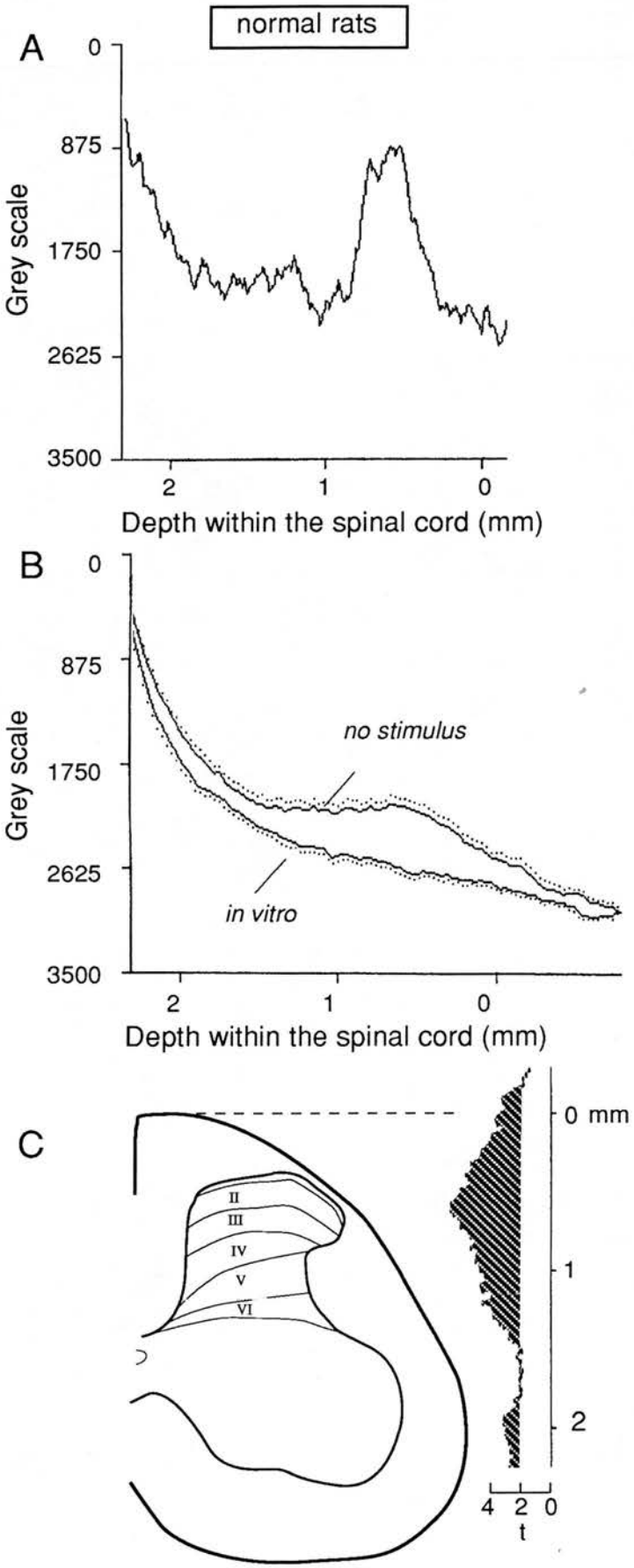
3/ whether impulses in primary afferents could evoke release.

A basal presence of a neuropeptide at a particular site in the nervous system is inferred by observing differences in the mean image analysis of microprobes not

inserted into the nervous system but simply incubated in the radiolabelled peptide (*in vitro*) and that of microprobes placed in the chosen area for a comparable time in the absence of any active stimulus and then incubated in the labelled peptide. With the latter it was relatively common in the present experiments for the autoradiographs to show a sharply defined zone of reduced binding [^{125} I]-NPY. The basal presence was determined by comparing the first 2-4 microprobes inserted into the spinal cord of 21 rats. *In vitro* microprobes used per experiment ranged from 1 to 11. Figure 9A is a density scan of such an image with a zone that extends from approximately 0.3mm to 0.9mm and peaks at a depth of approximately 0.6mm from the dorsal surface of the spinal cord. This zone includes most of the dorsal horn. Figure 9B compares the mean image analysis of 73 microprobes present in both sides of the spinal cord of the normal rat for 15 minutes in the absence of any active peripheral stimulus and that of 56 *in vitro* microprobes that had only been exposed to [^{125} I]-NPY and processed concurrently with those used *in vivo*. The zone of maximal difference between these two groups includes the zone of reduced binding in figure 9A but encompasses a broader area as not all autoradiographs produced such a focal zone. Figure 9C plots in 30µm intervals the differences between the mean image analyses of the two groups in relation to a schematic diagram of the lumbar spinal cord. The hatched area indicates where these differences are significant at the $P<0.05$ level ($t>2$) and this is down to a depth of 2.25mm from the dorsal surface of the spinal cord. Thus in normal animals, there was a significant basal release of ir-NPY throughout the entire dorsal horn that peaked at approximately 600µm from the dorsal surface of the spinal cord but also extended into the dorsal columns. This basal release was also present in the ventral horn but to a lesser extent.

Figure 9-The extensive basal presence of ir-NPY in the spinal cord of normal rats.

(A) is a density scan of a single autoradiographic image with a defined zone indicating failure of [^{125} I]-NPY to bind. This defined zone peaks at a depth of approximately 600μm from the dorsal surface of the spinal cord which corresponds to the peak in the middle of the dorsal horn in figure C. (B) The mean image analysis of two groups of microprobes are plotted with respect to depth within the spinal cord: those present in the spinal cord of drug naive rats for 15 minutes in the absence of any active peripheral stimulation (*no stim*, n=73) and those which are not inserted into the spinal cord but simply incubated in [^{125} I]-NPY (*in vitro*, n=56). For each mean image analysis the mean grey scale was determined in 30μm intervals and a line joins these points. At each analysis point the standard error of the mean (S.E.M.) is also plotted (+) for *no stim* and (-) for *in vitro*. (C) A plot of the 't'-statistics derived from the standard errors of the differences of the means at each analysis point in the mean image analyses shown in (B), is related to an outline of a transverse section of the lumbar spinal cord. The hatched area indicates where these sites are significant at the $P<0.05$ level.


normal rats
A
0
875
1750
2625
3500
Grey scale
2
1
0
Depth within the spinal cord (mm)
B
0
875
1750
2625
3500
Grey scale
no stimulus
in vitro
2
1
0
Depth within the spinal cord (mm)
C
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

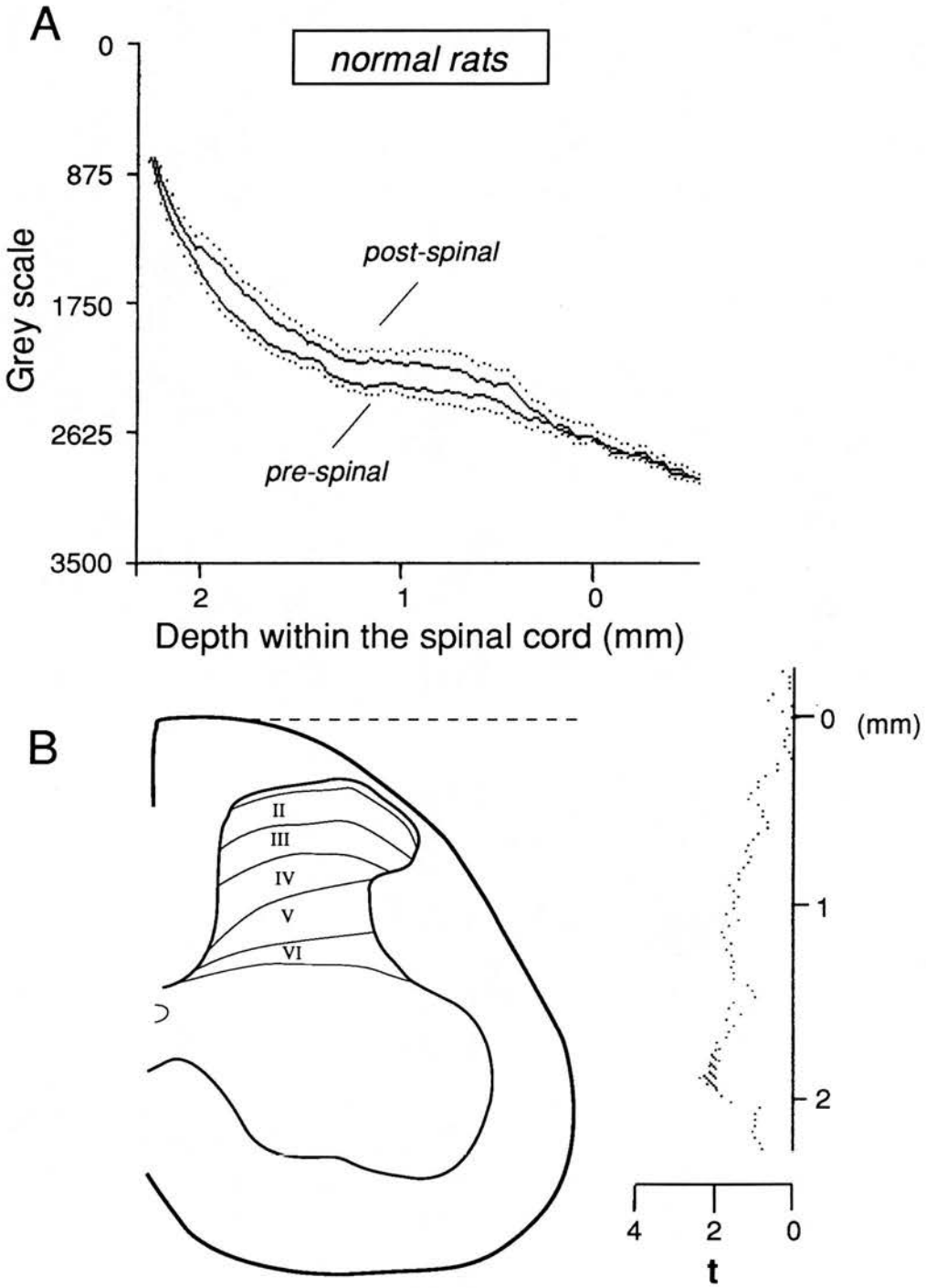
The mean image analysis of microprobes inserted into one side of the spinal cord in the absence of a peripheral stimulus might reasonably be expected to be comparable to those inserted into the opposite side. The two plots were found to be similar and there were no statistical differences between the two groups. Preliminary studies were also carried out to determine if the surgery necessary to isolate and mobilise the sciatic nerve preparatory to electrical stimulation changed the basal presence of extracellular ir-NPY. The mean image analysis plot of 38 microprobes remaining in the spinal cord in the absence of surgery to the sciatic nerve was similar to the mean image analysis of 58 microprobes remaining in the spinal cord after the sciatic nerve had been prepared for stimulation (not illustrated). This suggests that the additional surgery to isolate the sciatic nerve did not alter the basal presence of ir-NPY.

5.2.(d) Basal presence of ir-NPY in spinalised rats

The high levels of ir-NPY found in intact animals could represent release from the spinal terminations of fibres of supraspinal origin. To examine the contribution of such fibres, 4 rats were spinalised midway through the experiment to enable comparison of basal levels of ir-NPY before and after spinalisation. The range of microprobes used per rat prior to spinalisation was 6-13 and 2-8 after spinalisation. Figure 10A shows that the mean image analysis of 22 microprobes present in either side of the spinal cord after spinalisation is displayed above that of the mean image analysis of 35 microprobes present in either side of the spinal cord prior to spinalisation. This displacement is from 2.25mm to approximately 0.2mm from the dorsal cord surface, almost the entire length of the inserted microprobe. It suggests higher levels of extracellular ir-NPY and hence inhibition of release by the brainstem under normal conditions. These differences however are only significant in the lower ventral horn (laminae VII/VIII) as illustrated in figure 10B. This result thus

Figure 10. Spinalisation alters the release of ir-NPY in the ventral horn of the spinalized cord of normal rats.

The mean image analysis of 22 microprobes present for 15 minutes in both sides of the spinal cord of normal rats in the absence of any active peripheral stimuli, 1 hour after spinalisation (*post-spinal*) is displaced above that of 35 microprobes present in both sides of the spinal cord for 15 minutes in the absence of any active peripheral stimuli prior to spinalisation (*pre-spinal*). (B) The differences between the *post-spinal* and *pre-spinal* groups of microprobes are plotted with respect to an outline of the spinal cord.


A
normal rats
0
875
1750
2625
3500
Grey scale
post-spinal
pre-spinal
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0
(mm)
1
2
4
2
0
t

suggests that the basal release of ir-NPY in the dorsal horn is not under significant continuous supraspinal control.

5.2.(e) Peripheral nerve stimulation and the spinal release of ir-NPY

Although NPY cannot be significantly released directly from the terminals of primary afferent fibres in normal rats due to its virtual absence in dorsal root ganglion neurones, impulses in these fibres could result in intraspinal release (or inhibition of release) through activating intraspinal or supraspinal pathways. In microprobe experiments involving electrical nerve stimulation, a large peripheral nerve initially has been employed. In this study, the sciatic nerve was chosen as this is the nerve that was to be ligated in studies using the Bennett and Xie model to investigate the effect of peripheral nerve injury on the spinal release of ir-NPY. Both large and small diameter fibres of the sciatic nerve were stimulated as a possible means of influencing the basal release of NPY. Due to uncertainty on the persistence of any possibly released ir-NPY following peripheral stimulation, only one stimulation period of 15 minutes was used in each experiment during which 2 microprobes were present in the side of the spinal cord ipsilateral to the nerve that was stimulated. Since there is evidence that some neuropeptides are not rapidly degraded after in vivo release in the central nervous system (Duggan et al 1990, Hope et al 1990b) 6 microprobes were inserted into the ipsilateral side of the cord for a further three periods after electrical stimulation to determine if persistence following release occurred and to examine the decline in extracellular levels. Although attempts were made to standardise the time of insertion of microprobes after stimulation there was some variation due to the need to alter the sites of insertion. Thus all insertion times are presented as a range during which the microprobes were inserted into the spinal cord after stimulation. Three parameters of electrical stimulation were used, all with a pulse width of 0.5ms:
1/ A stimulus intensity of 1Hz at 3 times the threshold (T) to elicit a cord dorsum volley was used to stimulate large myelinated fibres.

2/ A stimulus intensity of 20Hz at 3 times threshold was used to stimulate large myelinated fibres.

3/ A stimulus intensity of 2Hz greater than 100 times threshold was used to activate both myelinated and unmyelinated fibres.

The basal presence was determined separately in each set of experiments. Two microprobes were inserted into each side of the spinal cord before stimulation since the levels of release of ir-NPY had been shown to be similar for either side. These 'control' microprobes formed the basis for assessing the possible effects of nerve stimulation. Thirty six microprobes were inserted into the spinal cord in the presence of peripheral nerve stimulation at 1Hz, 20Hz and 2Hz as detailed in the following pages.

5.2.(ei) Stimulation of large myelinated fibres at 1Hz

In 8 rats microprobes were inserted into the spinal cord before, during and after one 15 minute period of electrical stimulation at 1Hz (3 x T). The mean image analysis of 16 microprobes inserted into the spinal cord during large fibre stimulation at 1Hz is displaced below that of 26 microprobes inserted into the spinal cord prior to stimulation from a depth of approximately 1.9mm to 0.25mm from the dorsal cord surface as shown in figure 11(i)A. The differences between the two groups however, are not significant as illustrated in figure 11(i)B. The mean image analysis of 37 microprobes inserted into the spinal cord from 5-60 minutes following stimulation is compared to the mean image analysis of those microprobes inserted prior to stimulation (n=26) in figure 11(ii)A. The lower figure (11(ii)B) illustrates that the mean image analyses of the two groups are similar and show no significant differences. Thus the basal presence of ir-NPY was not altered by this form of electrical stimulation.

Figure 11. Failure of large myelinated fibre stimulation of the sciatic nerve at 1Hz to release ir-NPY in the ipsilateral spinal cord of normal rats.

11(i) microprobes present during stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord of normal rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, ipsi & contra* n=26) and those present in the spinal cord ipsilateral to the nerve during stimulation at 1Hz (3 x T, 0.05ms). This latter group is the mean of 16 microprobes (*stim 1Hz, ipsi*). A plot of the t-statistics derived from the standard errors of the differences of means at each 30μm interval is related to an outline of the lumbar spinal cord. The absence of hatched areas indicates that these differences are not significant at the $P<0.05$ level.

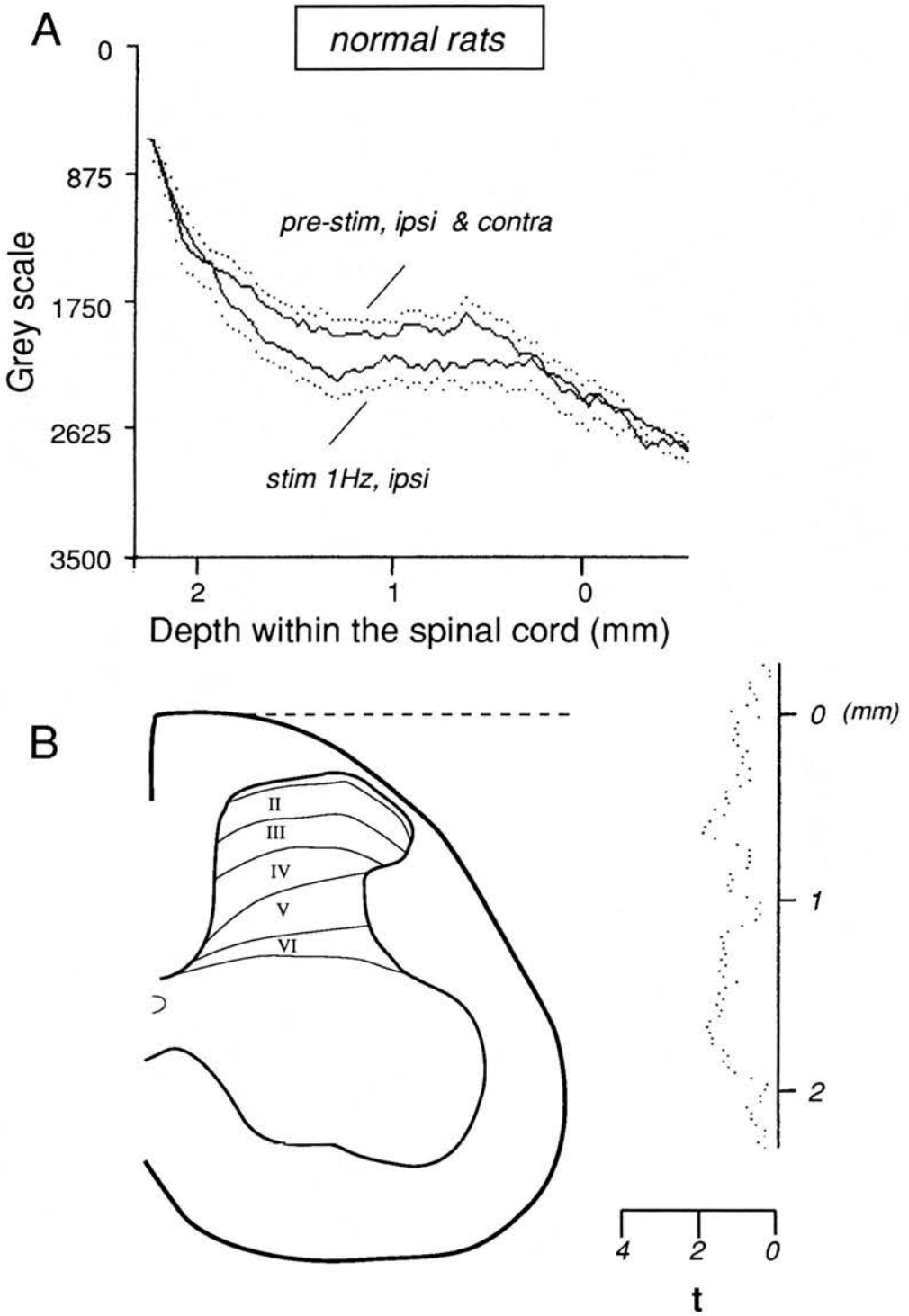
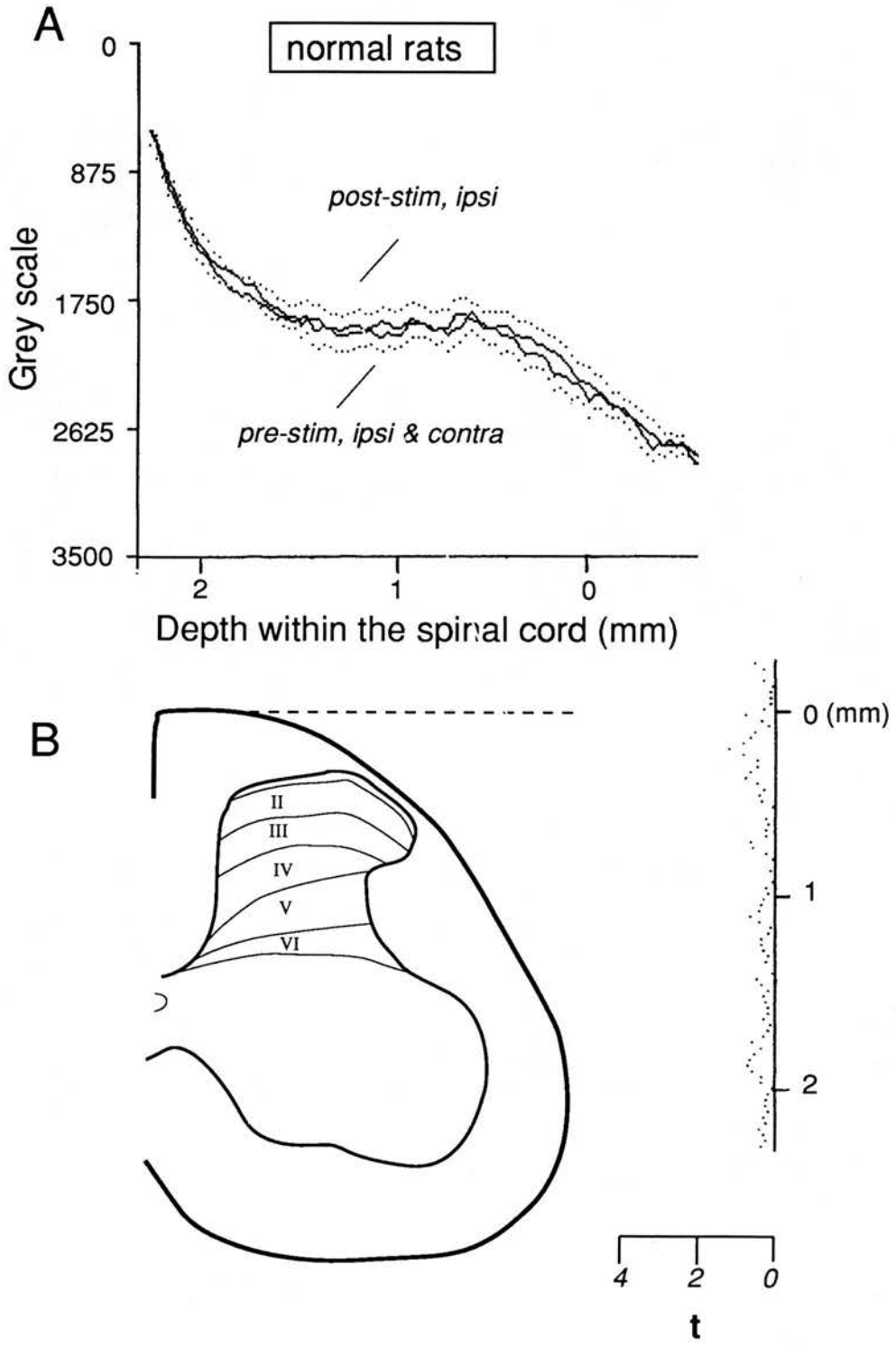

A
normal rats
0
875
1750
2625
3500
Grey scale
pre-stim, ipsi & contra
stim 1Hz, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

Figure 11. Failure of large myelinated fibre stimulation of the sciatic nerve at 1Hz to release ir-NPY in the ipsilateral spinal cord of normal rats

11(ii) microprobes inserted after stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord of normal rats for 15 minutes in the absence of any active peripheral stimuli are plotted: those present in both sides of the spinal cord prior to stimulation at 1Hz (*pre-stim, ipsi & contra* n=26*)* and those present in the ipsilateral spinal cord from 5-60 minutes following stimulation (*post-stim, ipsi* n=37*).* (B) The t-statistics show that the *pre-stim* and *post-stim* groups are not different.


A
normal rats
0
875
1750
2625
3500
Grey scale
post-stim, ipsi
pre-stim, ipsi & contra
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

5.2.(eii). Stimulation of large myelinated fibres at 20Hz

The effects of such stimulation were studied in six rats. The plot of the mean image analysis of 22 microprobes inserted into the spinal cord prior to stimulation is very similar to that of 11 microprobes present during stimulation at 20Hz as shown in figure 12(i)A. Thus stimulation of large fibres of the sciatic nerve at 20Hz failed to release ir-NPY (figure 12(i)B). The mean image analysis of 33 microprobes inserted into the ipsilateral spinal cord from 5-60 minutes following stimulation is displaced below those inserted prior to stimulation (n=22) in the region of 0.2-1.3mm from the dorsal cord surface as illustrated in figure 12(ii)A. These differences, however, are not significant at the $P<0.05$ level as shown in figure 12(ii)B.

Figure 12. Failure of large myelinated fibre stimulation of the sciatic nerve at 20Hz to release ir-NPY in the ipsilateral spinal cord of normal rats.

12(i) microprobes present during stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord of normal rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, ipsi & contra* n=22) and 11 microprobes present in the spinal cord ipsilateral to the nerve during stimulation at 20Hz (3 x T, 0.05ms, *stim 20Hz, ipsi*). A plot of the t-statistics derived from the standard errors of the differences of means at each 30μm interval is related to an outline of the lumbar spinal cord. There are no significant differences at the $P<0.05$ level (B).

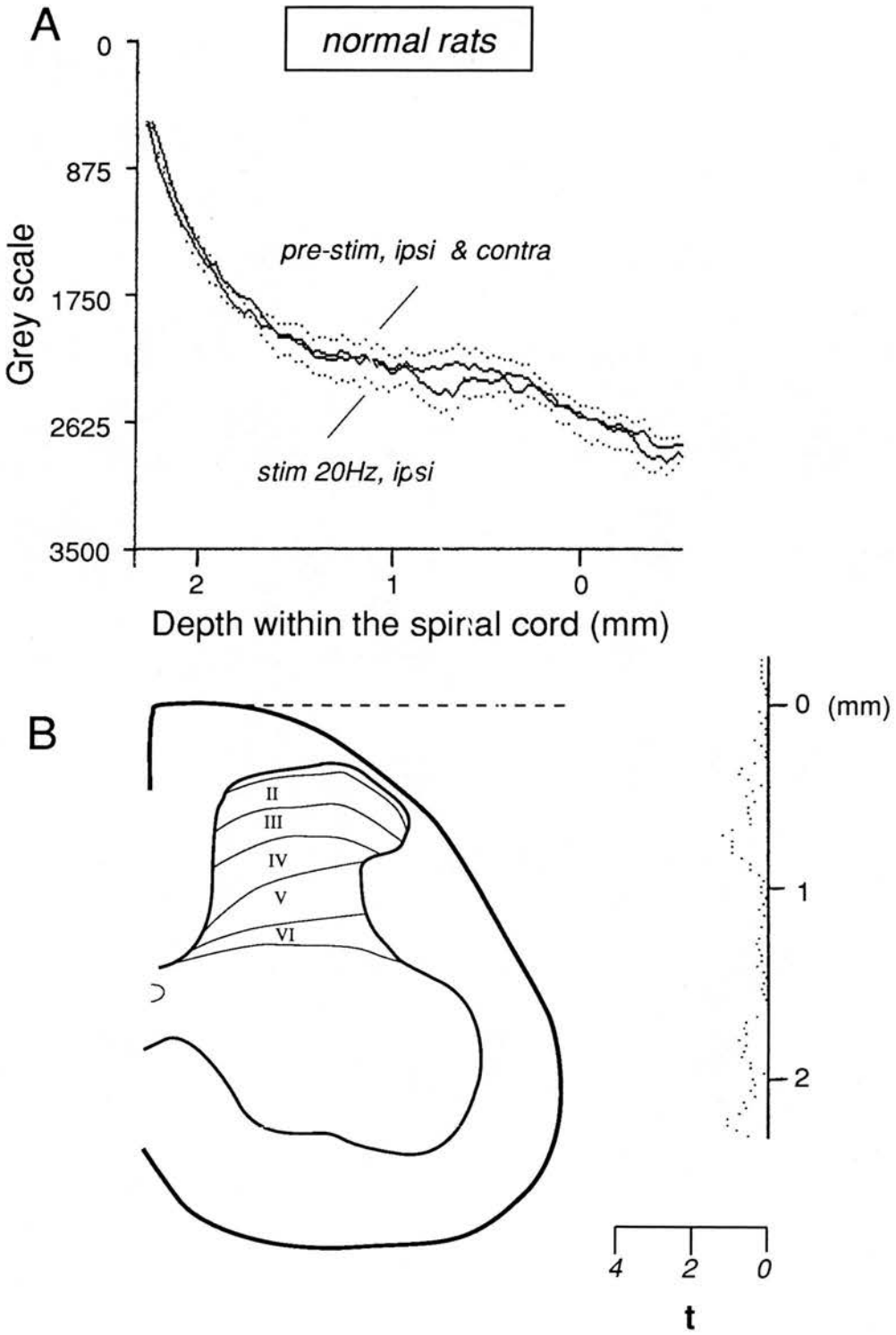
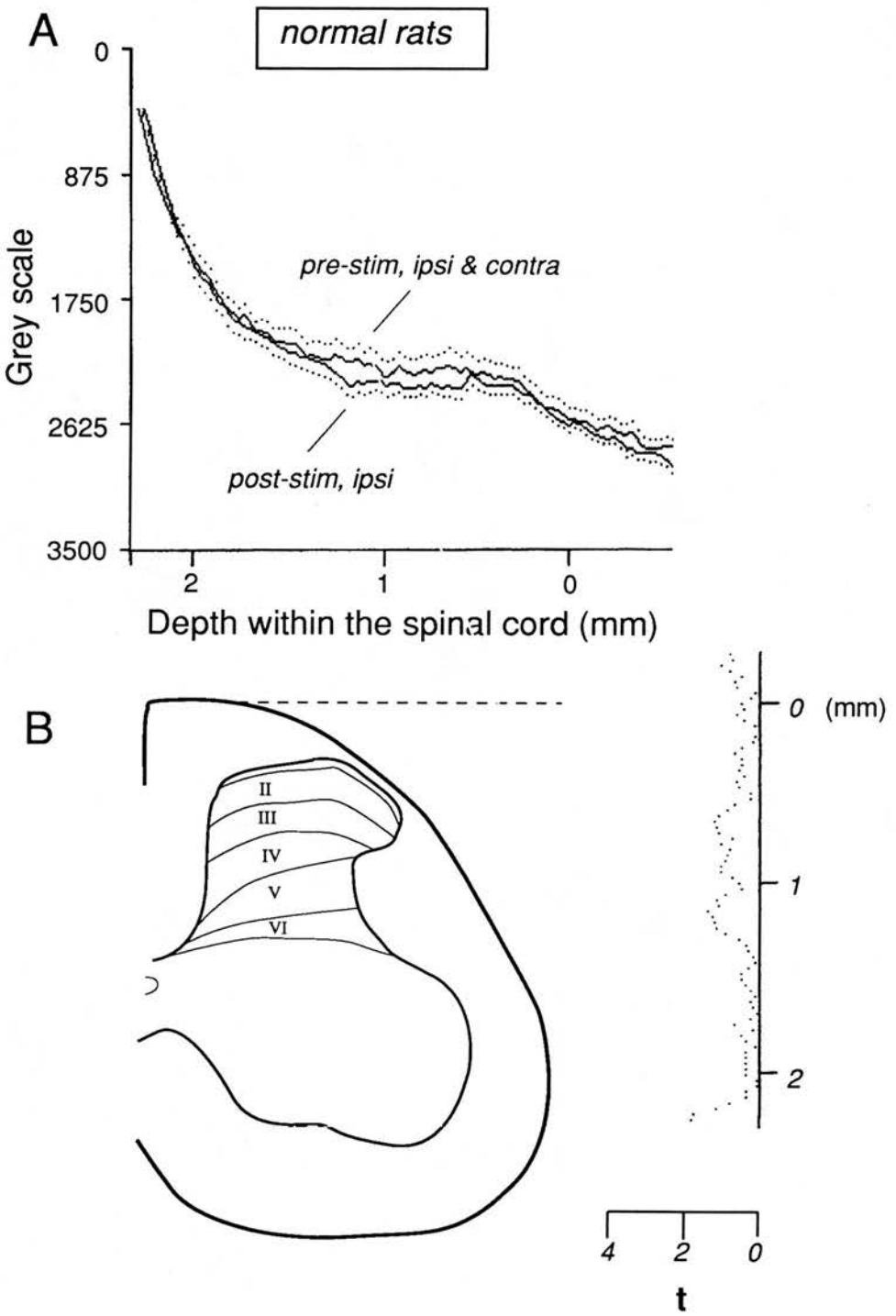

A
normal rats
0
875
1750
2625
3500
Grey scale
pre-stim, ipsi & contra
stim 20Hz, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

Figure 12. Failure of large myelinated fibre stimulation of the sciatic nerve at 20Hz to release ir-NPY in the ipsilateral spinal cord of normal rats following stimulation.

12(ii) microprobes inserted after stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord for 15 minutes in the absence of any active peripheral stimuli are plotted: those present in both sides of the spinal cord prior to stimulation at 20Hz (*pre-stim, ipsi & contra* n=22*)* and those present in the ipsilateral spinal cord from 5-60 minutes following stimulation (*post-stim, ipsi* n=33*)*. The *pre-stim* and *post-stim* groups are not statistically different as shown by the t-statistics (B).


A
normal rats
0
875
1750
2625
3500
Grey scale
pre-stim, ipsi & contra
post-stim, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

5.2.(eiii) Electrical stimulation of myelinated and unmyelinated fibres at 2Hz

Electrical stimulation of the sciatic nerve at 2Hz was studied in 6 rats. The plot of the mean image analysis of microprobes present in the spinal cord during nerve stimulation at 2Hz (n=12) is displaced above the plot of 24 pre-stimulus microprobes between approximately 0.5mm and 1.8mm from the dorsal surface of the spinal cord suggesting release that peaked in the upper ventral horn as shown in figure 13(i)A. At this zone of displacement, the differences between pre-stimulus and stimulus microprobes were close to significance at the $P<0.05$ level (figure 13(i)B). The mean image analysis of 34 microprobes present in the spinal cord from 5-65 minutes following stimulation are displaced above the mean plot of microprobes inserted prior to stimulation from almost 2mm to the dorsal cord surface as illustrated in figure 13(ii)A. Figure 13(ii)B shows that these differences are not significant.

Figure 13. Failure of myelinated and unmyelinated fibre stimulation of the sciatic nerve at 2Hz to release ir-NPY in the ipsilateral spinal cord of normal rats.

13(i) microprobes present during nerve stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord for 15 minutes were plotted with respect to depth within the spinal cord**:** 12 microprobes present in the spinal cord ipsilateral to the nerve during stimulation at 2Hz (>100 x T, 0.05ms, *stim 2Hz, ipsi*) are displaced above those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, ipsi & contra* n=24*)*. A plot of the t-statistics at each 30μm interval is related to an outline of the lumbar spinal cord. The absence of hatched areas indicates that these differences are not significant at the $P<0.05$ level although the t values are close to 2.

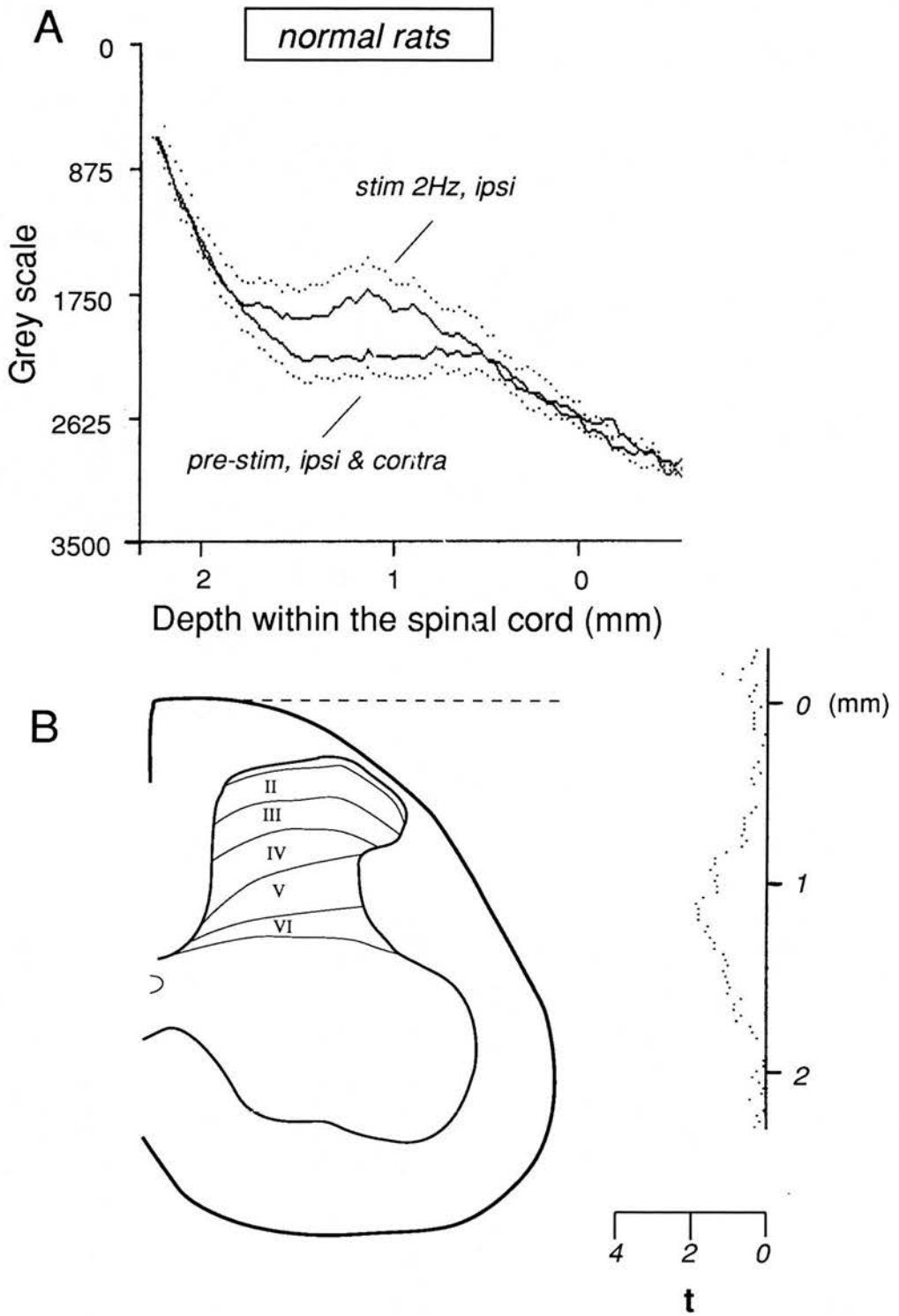
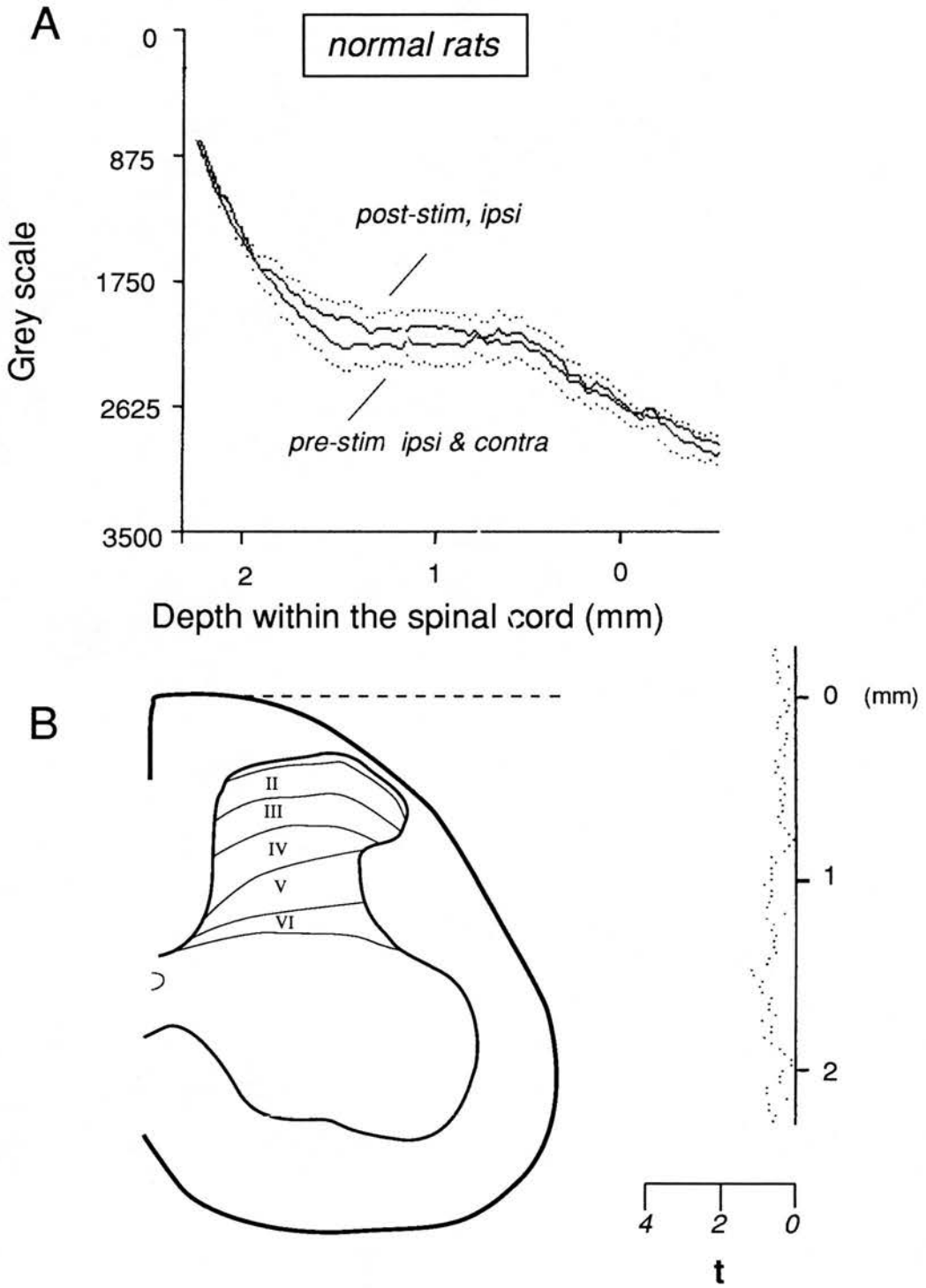

A
normal rats
0
875
1750
2625
3500
Grey scale
stim 2Hz, ipsi
pre-stim, ipsi & contra
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

Figure 13. Failure of myelinated & unmyelinated fibre stimulation of the sciatic nerve at 2Hz to release ir-NPY in the ipsilateral spinal cord of normal rats.

13(ii) microprobes inserted after stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord for 15 minutes in the absence of any active peripheral stimuli are plotted**:** those present in both sides of the spinal cord prior to stimulation at 2Hz (*pre-stim, ipsi & contra* n=24)and those present in the ipsilateral spinal cord from 5-65 minutes following stimulation (*post-stim, ipsi* n=34*).* (B) The t-statistics show that the two groups are not significantly different.


A
normal rats
0
875
1750
2625
3500
Grey scale
post-stim, ipsi
pre-stim ipsi & contra
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0
(mm)
1
2
4
2
0
t

5.3. DISCUSSION

The significant findings of this study which I shall discuss are:

(a) the presence of ir-NPY in the entire dorsal horn and ventral horn of the lumbar spinal cord of normal rats in the absence of any active peripheral stimulus.

(b) an alteration in the basal release of ir-NPY only in the deep ventral horn after spinalisation.

(c) the failure of nerve stimulation to significantly release ir-NPY.

Prior to discussing these results it is first necessary to consider the significance of the finding of a basal presence of ir-NPY in the absence of peripheral stimuli.

5.3.(a) Basal presence-real or artefact?

Release of ir-NPY from cells and fibres ruptured during the introduction of microprobes cannot be entirely excluded. Differing patterns of basal release however have been detected for different neuropeptides both in the spinal cord of the rat (Lang & Hope 1994; Hope et al, 1994; Riley et al, 1996) and the cat (Duggan et al, 1988a; Morton et al, 1989; Duggan et al, 1990; Schaible et al, 1994) despite the passage of microprobes through areas enriched in the relevant compound. Repeatedly placing microprobes in the same site might be expected to increase the probability of rupturing neuropeptide -containing structures. In the present study all microprobes were inserted into the area corresponding to the sciatic distribution in the spinal cord, but the sites of probe insertion within this area were varied in an attempt to minimise damage.

The antibodies immobilised to the microprobes of the present experiments were C-terminus directed and hence, as well as detecting NPY, it is possible that the shorter fragments resulting from enzymatic degradation might also bind to

microprobes. NPY appears to be relatively resistant to degradation and although degraded by some central peptidases little is known about the degradation of NPY within the spinal cord (Ludwig et al, 1995, see section 1.1.2.).. Although the immobilised antibodies used in the present experiments did recognise peptide YY, analysis of chromatographic and radioimmunoassay data led De Quidt and co-workers to conclude that NPY is the main if not the only, member of the pancreatic polypeptide family in the central nervous system of the rat, pig and human (De Quidt et al, 1990). Therefore, it is likely that the antibody microprobes inserted into the spinal cord of the rat predominantly detected NPY.

5.3.(b) Basal release-The stimulus for release?

A working definition of release is an increase in extracellular levels when a stimulus is delivered. In microprobe experiments, this is inferred by the difference between pre and intra stimulus groups. In this study, the basal presence of ir-NPY was determined by comparing the mean image analysis of in vitro microprobes (not inserted into the spinal cord but simply incubated in radioactivity) with those present in the spinal cord in the absence of any active peripheral stimulus. The differences between these two groups can also be called release as this basal presence is most likely due to continuous activity in some structure under the conditions of these experiments. There are several possibilities for such activity. Microprobe studies of release of neuropeptides in the spinal cord are performed in animals which have been anaesthetised and subjected to considerable surgery. There is no evidence that anaesthesia is a stimulus for the release of peptides. Despite general anaesthesia such animals could have a continued spinal input of impulses in nociceptors of both superficial and deep origin. This has been proposed as the source of the basal presence of ir-substance P in the superficial dorsal horn of cats (Duggan et al, 1988b)

and rats (Lang & Hope 1994) since substance P is readily released in this area by peripheral noxious stimuli and particularly as inflammation develops (Schaible et al, 1990). In contrast to substance P, NPY is not present in the primary afferents of normal rats. However, impulses in primary afferent fibres could result in intraspinal release (or inhibition of release) of NPY by activating intraspinal or supraspinal pathways.

In this study the sciatic nerve was stimulated in an attempt to release ir-NPY from the endings of primary afferents and to study the time course of this peptide if it persisted. However, electrical stimulation of both myelinated and unmyelinated fibres and large myelinated fibres alone failed to release ir-NPY at the frequencies chosen. Hence it is unlikely that the large basal presence of ir-NPY can be attributed to continued activity in peripheral nociceptors as a result of surgery. In the cat, noxious mechanical and thermal stimulation have failed to release ir-NPY (Mark et al, 1997). Thus although the present study indicates that ir-NPY is continually released in the spinal cord of the anaesthetised rat the stimulus for this release is unknown.

5.3.(c) The localisation of ir-NPY in the spinal cord and the possible sources of the extracellular release

Radioimmunoassay studies of tissue extracts have found higher levels of ir-NPY in the dorsal horn compared to the ventral horn in all species studied (Allen et al, 1984; Roddy et al, 1990; Gibson et al, 1984). Immunocytochemical studies have shown ir-NPY containing cells and terminals mainly in laminae I-III of the lumbrosacral spinal cord with lower levels around the area of the central canal and the ventral horn (Hunt et al, 1981a & b; Sasek & Elde 1985; DeQuidt & Emson 1986; Krukoff 1987, Leah et al, 1988).

This is in broad agreement with the results obtained in this study which found that ir-NPY was present throughout the dorsal horn, dorsal columns and ventral horn. It should be emphasised however, that microprobes detect the extracellular presence of ir-NPY and hence release from axon terminals as opposed to the levels in cell bodies and cell processes as determined by immunocytochemical means.

NPY immunoreactivity has not been found in primary afferent neurones but a proportion of intrinsic spinal neurones contain ir-NPY (DeQuidt & Emson 1986; Sasek & Elde 1985; Rowan et al, 1993; Todd & Spike 1993). A contribution from descending systems has been suggested due to the similar distribution pattern of noradrenergic fibres from the brainstem (Westlund et al, 1983). Colocalisation of ir-NPY in medullary catecholamine containing neurones has been described (Hokfelt et al 1983; Everitt et al, 1984; Blessing et al, 1987). However, only the C1 neurones of the medulla oblongata (adrenaline containing group of neurones) and the A6 neurones (noradrenaline containing) of the locus coeruleus have been found to contain spinally projecting neurones which were immunoreactive for NPY (Holet et al, 1988; Blessing et al, 1987).

To determine whether continued activity in descending fibres was contributing to the basal release of ir-NPY in the rat spinal cord, microprobes were inserted into the lumbar spinal cord one hour after spinalisation of the lower thoracic cord. The mean image analysis of microprobes inserted after spinalisation was displaced above those inserted prior to spinalisation. This suggests that the extracellular levels of ir-NPY were increased after spinalisation and that the descending fibres inhibit release under normal conditions. These differences were significant only in the lower ventral horn but not at any of the dorsal horn sites. Thus, the activity in descending fibres was not found to be a major source of the basal

presence of ir-NPY. This suggests that intrinsic spinal neurones were predominantly responsible for the basal presence.

5.3.(d) The wide distribution of this basal release

A surprising finding of the present study was the extent of the basal presence of extracellular ir-NPY which was present in significant levels throughout the entire dorsal horn and ventral horn.

Sites of neuropeptide presence can be inferred with great accuracy when dye is ejected from a single microprobe and its position in the spinal cord subsequently determined by spinal cord section. The location of sites of release is less precise when many microprobes are grouped and the average obtained as only one dye spot is produced per experiment. As microprobes are inserted a fixed distance into the spinal cord the spinal laminae may vary between individual animals and also with distance from the midline. These anatomical differences, may contribute to the broadness of the sites of release obtained from the mean image analyses of many microprobes but they cannot explain the differences between NPY and other neuropeptides studied in the rat such as substance P (Lang & Hope 1994, Duggan et al, 1988b), dynorphin (Riley et al, 1996) and galanin (Morton et al, 1989; Hope et al, 1994).

The ability of a neuropeptide to resist rapid degradation following release is an important determinant of its ability to access receptors and influence neurones remote from sites of release (Fuxe et al, 1990; Liu et al, 1994; Beck et al, 1995). Little is known of the degradation of NPY in the central nervous system but from its structure of a polyproline like helix lying antiparallel to an amphiphilic alpha-helix together with an amidated C terminus, NPY has been proposed to be relatively resistant to endopeptidase and carboxypeptidase attack (Ludwig et al, 1995). Thus the wide distribution of basal extracellular ir-NPY may represent wide diffusion rather

than release from a multiplicity of sites. Thus, the finding of a basal presence of ir-NPY in the dorsal and ventral white matter may also relate to diffusion after release.

High affinity NPY binding sites have been found on cultured dorsal root ganglion neurones and autoradiographic studies have suggested that NPY receptors are located on primary afferent terminals concentrated in laminae II of the spinal cord (Walker et al, 1988; Kar & Quirion 1992). Mantyh and co-workers found binding sites in laminae I, II, V, X and Onufs nucleus (Mantyh et al, 1994). NPY receptor mRNA has been described in 20% of dorsal root ganglion neurones of normal rats. These have been found mainly in small dorsal root ganglion neurones with only a few medium and large neurones labelled (Jazin et al, 1993b, Zhang et al, 1994a). Zhang and co-workers have suggested that both Y1 and Y2 receptors are synthesised in dorsal root ganglion neurones Y2 being transported to nerve terminals and Y1 being incorporated into somatic plasmalemma. The widespread release of NPY may allow this neuropeptide to act on the many receptors located throughout the spinal cord.

An unusual finding in the present study was the detection of ir-NPY in the perfusate just above the dorsal surface of the spinal cord. This may have simply resulted from the diffusion from the spinal cord but release from the meninges requires consideration. The spinal pia mater receives a rich innervation of small sensory axons via the ventral roots. These ventral root afferents are thought to be involved in the control of the pia blood vessels (Risling et al, 1994). Fibres immunoreactive to NPY, cGRP and SP have been found in the lumbrosacral pia mater lining the spinal cord (Risling et al, 1985; Shen et al, 1994). The rupturing of the pia mater by microprobes may contribute to the presence of ir-NPY which was detected beyond the surface of the perfusate.

5.3.(e) Functional implications of this study

The extensive distribution of extracellular ir-NPY suggests that NPY has an important role in spinal cord function. As previously described, NPY has been found to inhibit transmitter release both peripherally and centrally (see sections 1.2.4. & 1.2.6.). How these actions apply to events in the spinal cord *in vivo* is currently unknown.

CHAPTER 6: Studies of the Spinal Release of Neuropeptide Y in neuropathic rats

6.0 INTRODUCTION

The model of Bennett & Xie results in a partial loss of fibres distal to the site of injury. This injury differs from transection where the axotomy is complete thus there is no afferent input from the periphery. It has been suggested that an interaction between impulses from the periphery and the altered central state is responsible for the abnormal behavioural changes associated with this model (see sections 2.6. & 2.7). After peripheral nerve injury, NPY has been shown by in situ hybridisation studies to be synthesised in medium to large dorsal root ganglion neurones (Nahin et al, 1994; Kashiba et al, 1994).

The aim of this study was to examine the basal release of ir-NPY and the effect of peripheral nerve stimulation in rats with a unilateral constriction injury of the sciatic nerve. Sham experiments were performed in separate animals as controls. This enabled the basal release in the ipsilateral and contralateral cord with respect to the ligature to be compared in the neuropathic rat. It was of interest to determine whether the newly synthesised NPY could be released from the central terminals of primary afferents and whether release from these afferents was at different sites in the spinal cord compared to the basal release in normal rats. Using the antibody microprobe technique, the basal release of ir-NPY has been found to arise mainly from intrinsic neurones. To determine whether impulses in primary afferents were necessary to alter the basal presence of this peptide primary afferents in the sciatic nerve proximal to the ligature were stimulated. To investigate whether persistence occurred microprobes were inserted into the spinal cord for up to an hour following stimulation.

6.1. MATERIALS AND METHODS

6.1.(a) Microprobe preparation

Antibody microprobes were prepared as previously described, using a polyclonal antiserum (Peninsula Laboratories Europe Ltd) that had been raised in rabbits against the C terminal end of neuropeptide Y.

6.1.(b) Experimental preparation

A total of 41 male Wistar rats (weight range 190 to 350g when ligated; Charles River Ltd, UK) were used in this study. Anaesthesia was induced and the animal prepared as previously described (section 3.1.(b)). Before, during and after peripheral stimulation of the sciatic nerve, microprobes were inserted into both sides of the cord at a level determined by the electrophysiological recordings corresponding to the site of termination of the sciatic nerve. Only one stimulation period of 15 minutes (either 20Hz, 3 x threshold or 2Hz, >100 x threshold) was used per experiment and stimulation was always to the ipsilateral nerve proximal to the ligature site. Following electrical stimulation, microprobes were inserted into the spinal cord for three periods of no stimulation.

6.2. RESULTS

A total of 390 microprobes coated with antibodies to neuropeptide Y were inserted into the rat spinal cord form the basis of this analysis. 205 *in vitro* microprobes were prepared concurrently with those used *in vivo*. An additional 369 microprobes were used for *in vitro* sensitivity tests.

6.2.(a) *In vitro* tests

Prior to use and throughout use *in vivo*, the sensitivity of the prepared antibody microprobes were tested (as outlined in section 4.3).

6.2.(b) Behavioural results

Post-operatively, the International Association for the Study of Pain guidelines for the care of experimental animals were followed (Zimmermann et al, 1983). The animals used displayed the changes associated with this model such as hopping, licking, everted paw and ventroflexed toes (Bennett & Xie 1988).

Mechanical allodynia was tested by touching the skin with the standard Semmes-Weinstein set of Von Frey hairs (Stoelting, Wood Dale Illinois). These hairs are a series of nylon monofilaments (of increasing stiffness) that exert defined levels of force when pressed to the point where they bend against a hard and level surface. Increasing stiffness is achieved by increasing the hair's diameter thus the amount of force per unit area (Newtons) changes as grams of force increases. However, as the skin has non-trivial elasticity it is impossible to determine the exact contact area and thus the force was recorded in grams as described by Tal & Bennett (1994).

Animals were placed in cages with a perforated metal sheet that allowed mechanical stimuli to be applied to the hindpaw from below. The hairs were applied perpendicular to the mid-plantar glaborous skin of the rats hind paw and depressed slowly until they bent. Both hind paws were tested prior to surgery and then at regular intervals during the two weeks following surgery. Von Frey Hairs were tested in order of increasing force with each applied 5 times to slightly different loci within the area tested. The first hair to evoke a reflexive withdrawal was designated the threshold force. The mean paw withdrawal threshold pre-operatively for the left & right hindpaw were comparable. A significant decrease occurred in the threshold force of the paw ipsilateral to the nerve injury (the right paw) 3-5 days

postoperatively, 4.5 ± 0.4g (SEM), which continued to decrease throughout the time course studied with a mean of 3.4 ± 0.4g at 6-9 days postoperatively. On the experimental day, the paw withdrawal threshold for the right hindpaw was 3.3 ± 0.6g which was significantly smaller than the pre-operative mean. The threshold force of the contralateral paw also decreased (see 6(i) & 14(i)). 3-5 days postoperatively the mean paw withdrawal threshold for the left paw was 38.6 ± 2.3g which was significantly smaller from the preoperative value of 45.9 ± 2.8g ($P<0.05$). In addition, there was only a slight increase towards the control values over the next two time periods. The mean paw withdrawal threshold for the left paw was 39.2 ± 2.3g at days 6-9 and 39.7 ± 2.0g at 10-14 days postoperatively (see 6(i)). These means were also significantly smaller from the preoperative mean paw withdrawal threshold for the left paw ($P<0.05$). With sham operated rats there was no statistical difference between the sham operated paw (right) and normal paw (left) at any time point studied.

Mechano-hyperalgesia was assessed with pinprick. The point of a safety pin was used to indent but not penetrate the skin . The duration of the normal pin-prick-evoked hindpaw withdrawal was arbitrarily assigned a duration of 0.5 seconds as it was too short to time accurately. A measurement cut-off of 30 seconds was applied to the long-duration withdrawals seen ipsilateral to the nerve injury. The withdrawal duration of the ipsilateral paw peaked at 3-5 days after injury with a mean paw withdrawal latency of 20 ± 2s followed by a slight decrease over the next two time periods (see 6(ii) & 14(ii)). All post-operative means for the right (injured) paw were significantly greater than the pre-operative mean obtained for both the right and left paw ($P<0.001$). There was no change in the withdrawal duration of the contralateral hindpaw (see 6(ii)). With sham operated rats, there was no statistical difference between the sham operated paw and the normal paw at any time point studied.

Table 6 (i). Paw withdrawal threshold before and after partial nerve injury.

The mean paw withdrawal threshold (PWT) of the right (ipsilateral) paw, measured in grams (g), and left paw (contralateral) paw were compared before and following partial nerve injury. The surgical procedure was always performed on the right paw and the normal left paw served as a control.

* mean PWT for the right paw was significantly smaller than the mean for the left paw at $P<0.001$ level using paired students t-test.

• postoperative mean for the left paw was significantly smaller than the preoperative mean for this paw at $P<0.05$ level using unpaired students t-test.

Tble 6(ii). Paw withdrawal latency before and after partial nerve injury.

The mean paw withdrawal latency (PWL) of the right paw, measured in seconds (secs), and left paw were compared before and following partial nerve injury. The duration of the normal paw was arbitrarily assigned a duration of 0.5 seconds.

*mean PWL for the right paw is significantly larger than the mean for the left paw at $P<0.001$ level using paired students t-test.

BEHAVIOURAL RESULTS

6(i)-PAW WITHDRAWAL THRESHOLD BEFORE AND AFTER PARTIAL NERVE INJURY

Days Postoperatively	MEAN PWT (g) right ± SEM	MEAN PWT (g) left ± SEM
0	46.8 ± 2.8	45.9 ± 2.8 (n= 51)
3-5	4.5 ± 0.4 *	38.6 ± 2.3 • (n= 31)
6-9	3.4 ± 0.4 *	39.2 ± 2.3 • (n=29)
10-14	3.3 ± 0.6 *	39.7 ± 2.0 • (n=36)

6(ii)-PAW WITHDRAWAL LATENCY BEFORE AND AFTER NERVE INJURY

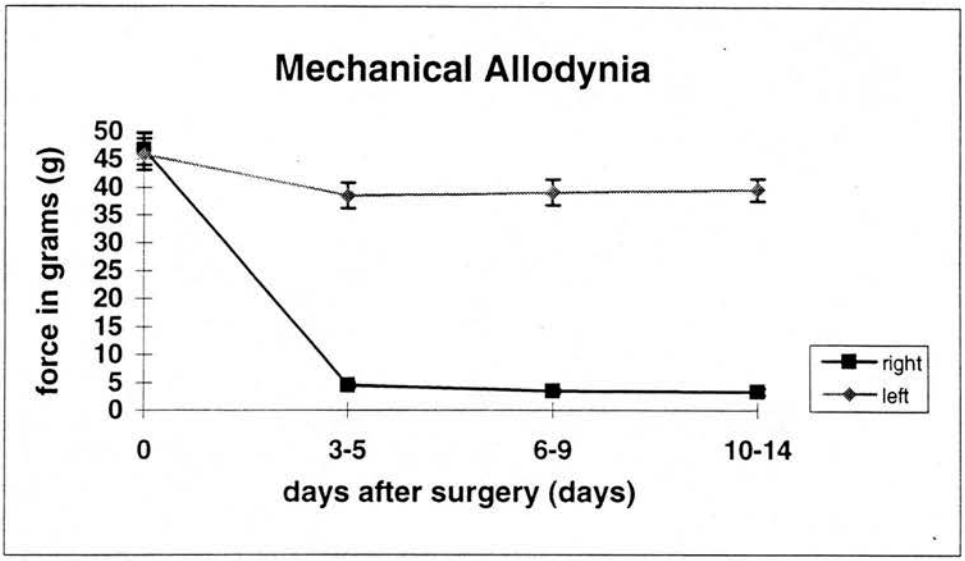
Days Postoperatively	MEAN PWL (secs) right ± SEM	MEAN PWL (secs) left ± SEM
0	0.5 ± 0	0.5 ± 0 (n= 21)
3-5	20 ± 2 *	0.5 ± 0 (n= 29)
6-9	19± 3 *	0.5 ± 0 (n= 13)
10-14	18 ± 2 *	0.5 ± 0 (n= 36)

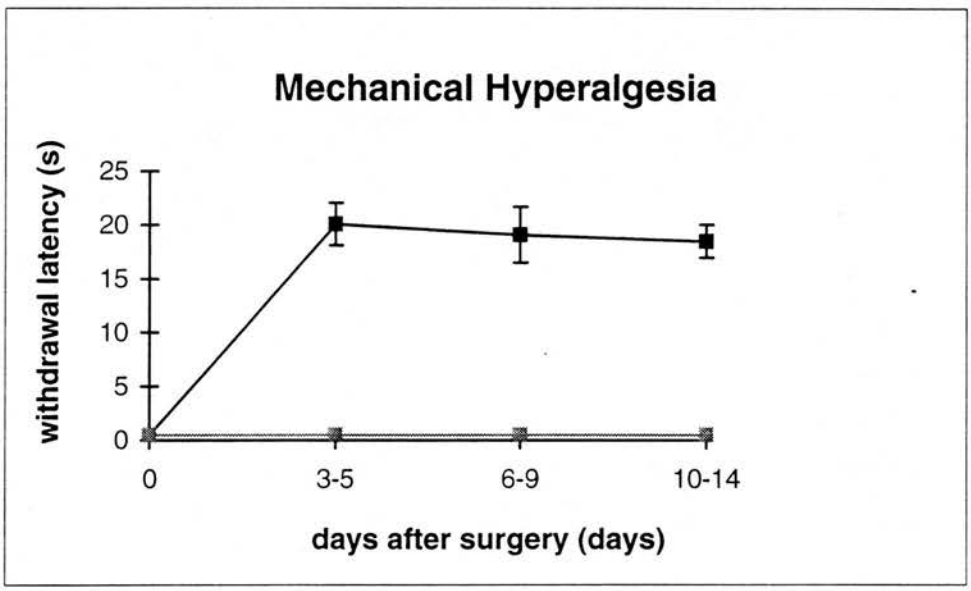
Figure 14(i). Behavioural testing for mechanical allodynia

The mean paw withdrawal threshold (PWT) of the right (injured) paw was compared with the left control paw. From 3-5 days postoperatively, the mean PWT for the right paw was reduced. Thus, from this time period until the experimental day the mean PWL of the right paw was significantly smaller than the mean for the left paw using student's paired t-test. These results are presented in table 6(i).

Figure 14(ii). Behavioural testing for mechanical hyperalgesia

The mean paw withdrawal latency (PWL) of the right (injured) paw was compared with the left control paw. From 3-5 days postoperatively, the mean PWL for the right paw was increased. Thus, from this time period until the experimental day the right mean PWL was significantly larger than the mean for the left paw using student's paired t-test. These results are presented in table 6(ii).


Mechanical Allodynia
force in grams (g)
50
45
40
35
30
25
20
15
10
5
0
0
3-5
6-9
10-14
days after surgery (days)
right
left


Mechanical Hyperalgesia
withdrawal latency (s)
25
20
15
10
5
0
0
3-5
6-9
10-14
days after surgery (days)

6.2.(c) Histology of injured nerve

Morphological studies of the ligated sciatic nerve have found a near complete loss of large myelinated fibres and a significant degeneration of small myelinated fibres distal to the ligature with relative sparing proximal to the lesion at 2 weeks postinjury (Basbaum et al, 1991; Munger et al, 1992; Coggeshall et al, 1993). The ligatured and normal sciatic nerves were removed from each animal at the end of the experiment. Sections of the normal and the ligated nerves were treated with solochrome cyanin which stains for myelin. This was performed by the histology laboratory of the department of Preclinical Vet. Science. The two sections shown in figure 15 illustrate the loss of myelin which occurs in the distal sciatic nerve after nerve injury.

6.2.(c) Spontaneous release of ir-NPY and sham operated rats

A basal presence of ir-NPY in the spinal cord of sham operated rats was inferred by comparing the mean image analysis of microprobes not inserted into the nervous system but simply incubated in radiolabelled NPY with microprobes inserted into the lumbar spinal cord of the sham animals in the absence of any active peripheral stimuli. The range of *in vivo* microprobes used was 7-13 per animal and for *in vitro* microprobes 4-7 per animal. Figure 16A shows that the mean image analysis of 42 microprobes inserted into either side of the spinal cord of sham animals is displaced above that of 23 *in vitro* microprobes derived from 4 sham rats. This displacement is from the tip to 2.50mm from the tip corresponding to the entire length of the microprobe within the spinal cord and beyond. Figure 16B illustrates the differences between the mean image analyses of the two groups in relation to a schematic diagram of the lumbar spinal cord. The hatched area indicates where the differences plotted in 30μm intervals are significant at the $P<0.05$ level ($t>2$). These differences are significant throughout laminae I-V of the dorsal horn, the dorsal columns and also

Figure 15. Histological sections of the sciatic nerve.

The sciatic nerve was removed from the animal, fixed in 10% formal saline and then wax-embedded. 8μm transverse sections were cut with a microtome and the sections treated with solochrome cyanin to stain for myelin and counterstained with eosin. A loss of large and small myelinated fibres after nerve injury has been reported by several studies. In this study a change in the myelin staining was found distal to the ligature compared to the non-ligatured nerve. (A) shows a section of the non ligatured sciatic nerve with the abundant myelin stained deep purple. (B) shows a section of the nerve distal to the ligatures. There is extensive loss of myelinated fibres in association with marked oedema.

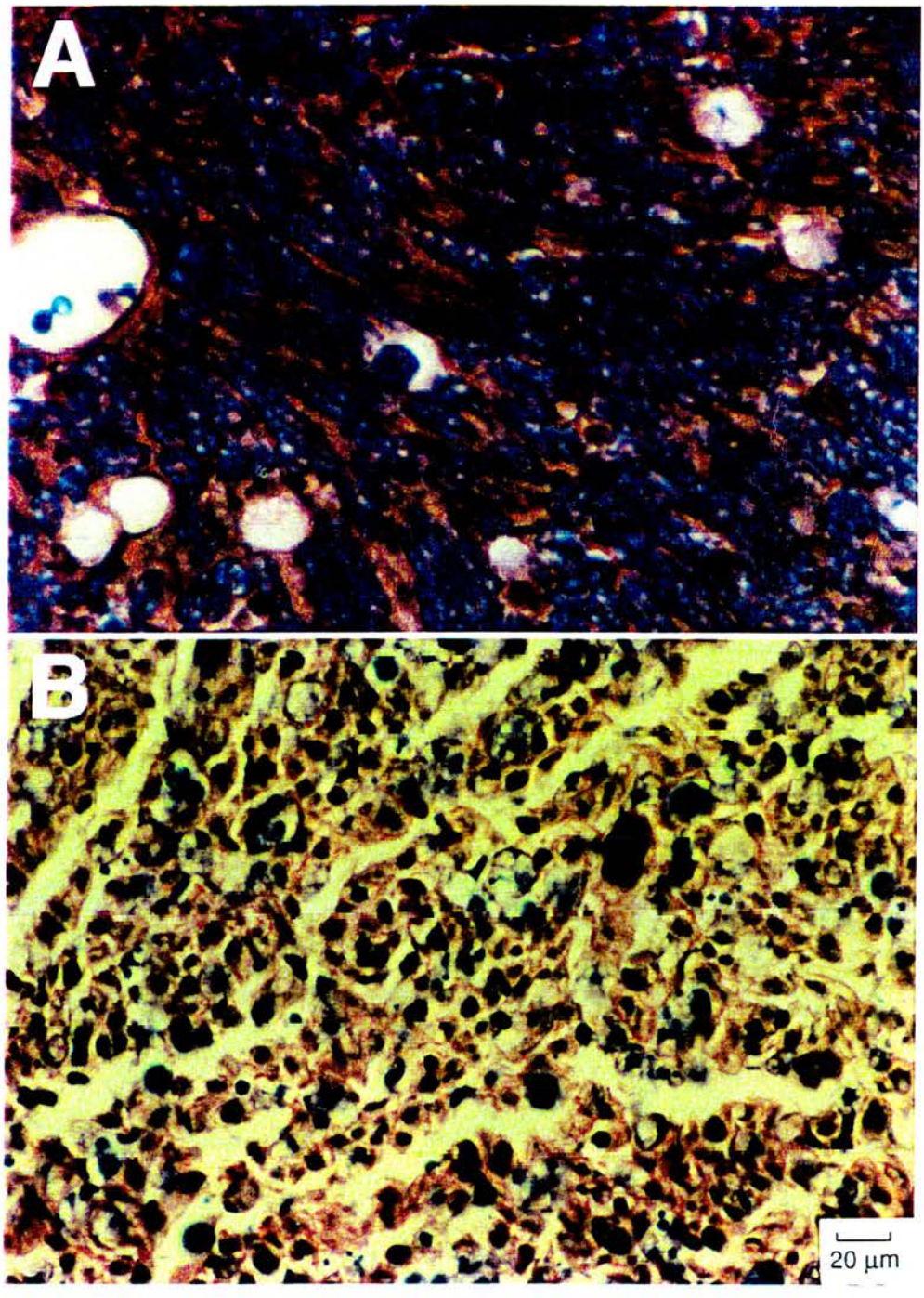
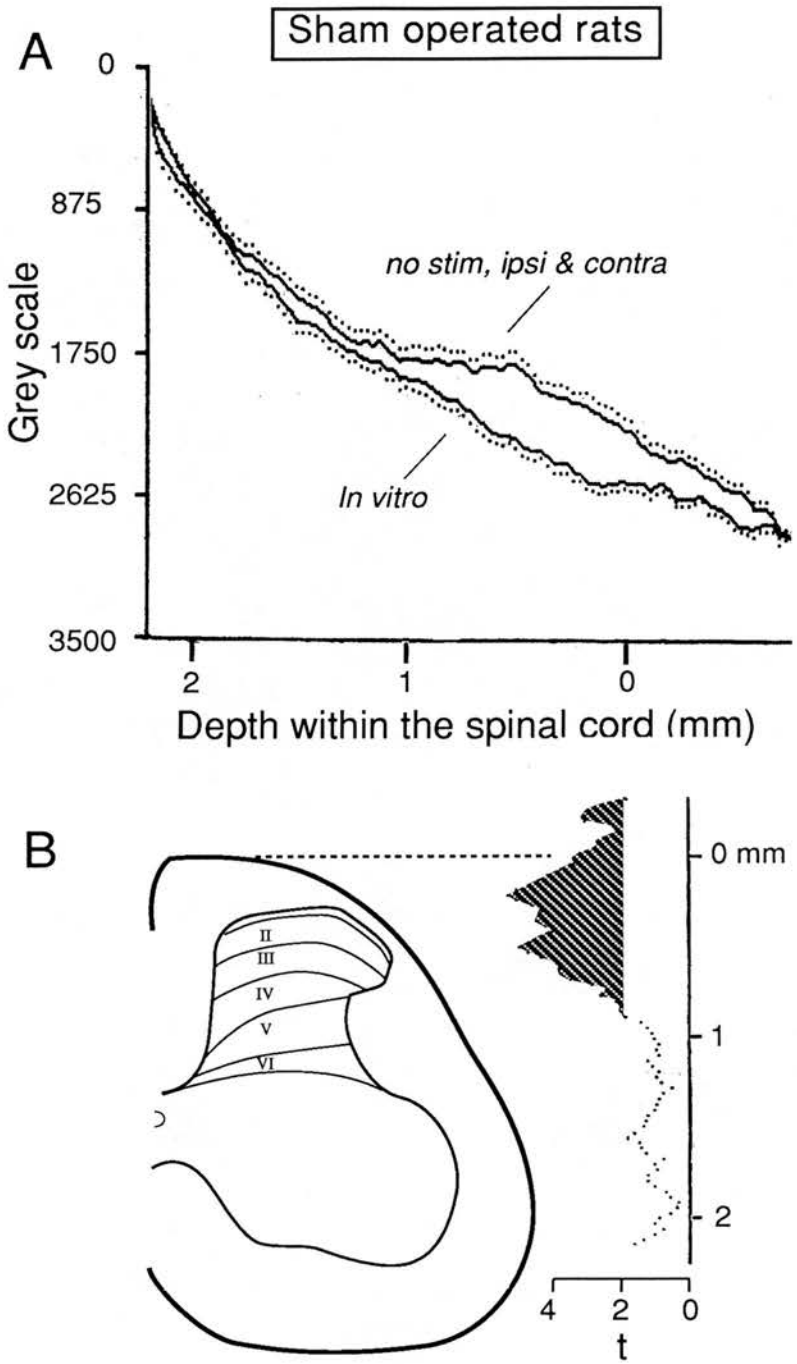

A
B
20 µm

Figure 16. The basal presence of ir-NPY in the spinal cord of sham operated rats.

(A) The mean image analysis of two groups of microprobes are plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord of sham operated rats for 15 minutes in the absence of any active peripheral stimulation (*no stim, ipsi & contra* n=42) and those which are not inserted into the spinal cord but simply incubated in [^{125} I]-NPY (*in vitro*, n=23). For each mean image analysis the mean grey scale was determined in 30μm intervals and a line joins these points. At each analysis point the standard error of the mean (S.E.M.) is also plotted (+) for *no stimulus* and (-) for *in vitro*. (B) A plot of the 't'-statistics derived from the standard errors of the differences of the means at each analysis point in the mean image analyses shown in (A), is related to an outline of a transverse section of the lumbar spinal cord. The hatched area indicates where these sites are significant at the $P<0.05$ level.

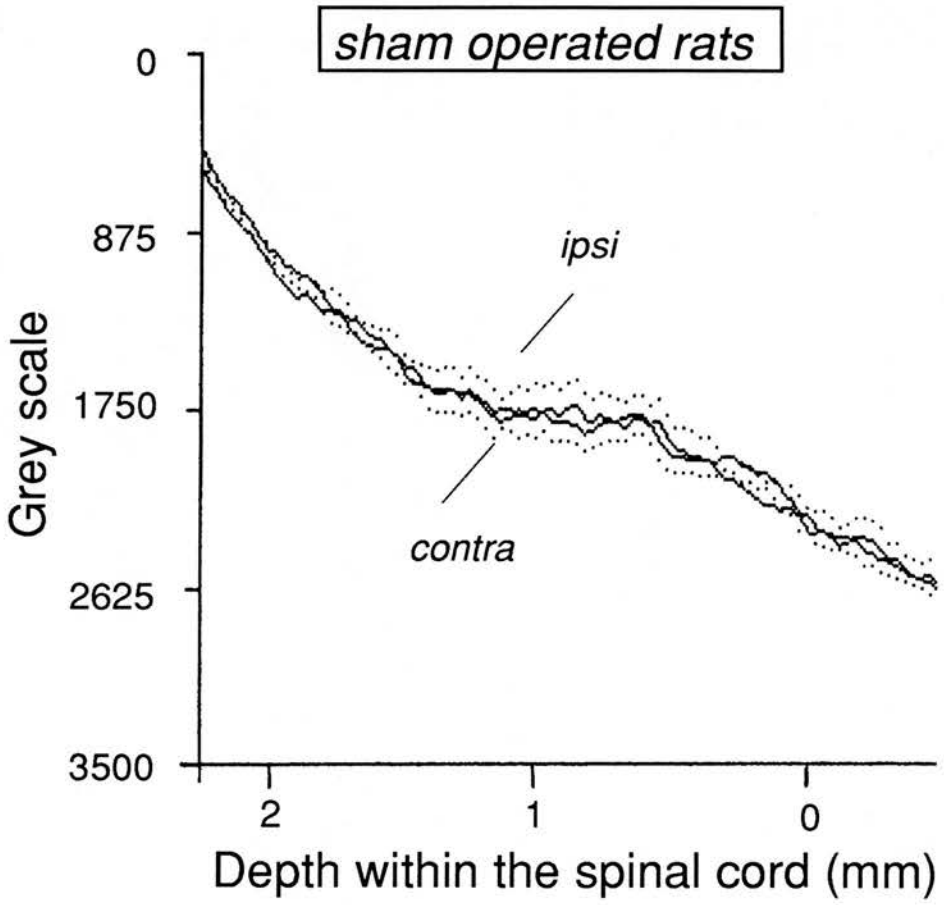

A
Sham operated rats
0
875
1750
2625
3500
Grey scale
no stim, ipsi & contra
In vitro
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

the superfusate of the spinal cord for 0.3mm. The zone of most significant release of ir-NPY approximates to laminae I, II and III of the dorsal horn.

It was important to determine whether exposure and manipulation of one sciatic nerve (the sham operation) produced differences in the basal levels of extracellular ir-NPY on the two sides of the spinal cord. The mean image analysis of microprobes inserted into the contralateral side (n=23) and into the ipsilateral side of the spinal cord of animals subjected to the sham operation (n=19) are compared in figure 17A. No significant differences were found between the two groups (see figure 17B).

Figure 17. The basal presence of ir-NPY in both sides of the spinal cord of sham operated rats is comparable.

The mean image analysis of two groups of microprobes present in the spinal cord for 15 minutes in the absence of any active peripheral stimuli were plotted with respect to depth within the spinal cord: those present in the spinal cord ipsilateral to the sham operation (*ipsi*, n=19) and those present in the contralateral spinal cord of the same animals for 15 minutes (*contra*, n=23). These plots are similar thus there are no statistical differences at the $P<0.05$ level.


sham operated rats
0
875
1750
2625
3500
Grey scale
ipsi
contra
2
1
0
Depth within the spinal cord (mm)

6.2.(d). Basal presence of ir-NPY in nerve ligated rats

In figure 18A the mean image analyses of microprobes inserted into the contralateral spinal cord of sham operated rats and that of microprobes inserted into the contralateral spinal cord of unilaterally nerve ligated rats are compared. In this figure, the mean image analysis of the nerve ligated rats is displaced above that of the sham operated rats from approximately 1.5mm to 0.25mm from the dorsal cord surface. Although this suggests increased basal levels of extracellular ir-NPY in the nerve ligated rats none of these differences are significant at the $P<0.05$ level as shown in figure 18B. When comparisons were made between the ipsilateral side of the spinal cord in sham operated and nerve ligated animals, significant differences were found. Figure 19A shows that the plot of the mean image analysis of microprobes inserted into the spinal cord ipsilateral to the nerve ligation is displaced above that of microprobes inserted into the sham operated rats. The displacement is from approximately 2.15mm to 0.3mm from the dorsal surface. Figure 19B illustrates that these differences are significant from 0.5mm to 1.5mm from the dorsal surface with a broad peak of greatest difference from 0.7mm to 1.1mm from the dorsal surface. The latter zone approximates to laminae IV, V, VI of the dorsal horn and the total area of significance includes laminae III, IV, V, VI and upper VII. Thus there was an additional zone of release in nerve ligated animals compared to sham operated animals and this difference was also apparent when the two sides of the spinal cord were compared in the nerve ligated rats.

Microprobes were inserted into the spinal cord of 10 rats to determine the basal presence of each side of the cord. Figure 20A shows the plot of the mean image analyses of 53 microprobes inserted into the spinal cord ipsilateral to the nerve ligation is displaced above the mean image analysis of 55 microprobes inserted into the opposite side of the spinal cord. The number of microprobes used per animal ranged from 7-14. Figure 20B illustrates that these differences are significant from 0.6 to 1.3mm from the dorsal surface which approxiamtes to laminae III, IV ,V & VI.

Figure 18. The basal presence of ir-NPY in the contralateral spinal cord of sham operated and nerve ligated rats is comparable.

(A) The mean image analysis of 23 microprobes present for 15 minutes in the contralateral side of the spinal cord of the sham operated animal in the absence of any active peripheral stimuli (*sham operated rats, contra)*, is displaced below that of 55 microprobes present for the same time period in the contralateral side of the spinal cord of the nerve ligated rats in the absence of any active peripheral stimuli (*nerve ligated rats, contra)*. (B) The differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. These differences are not significant at the $P<0.05$ level.

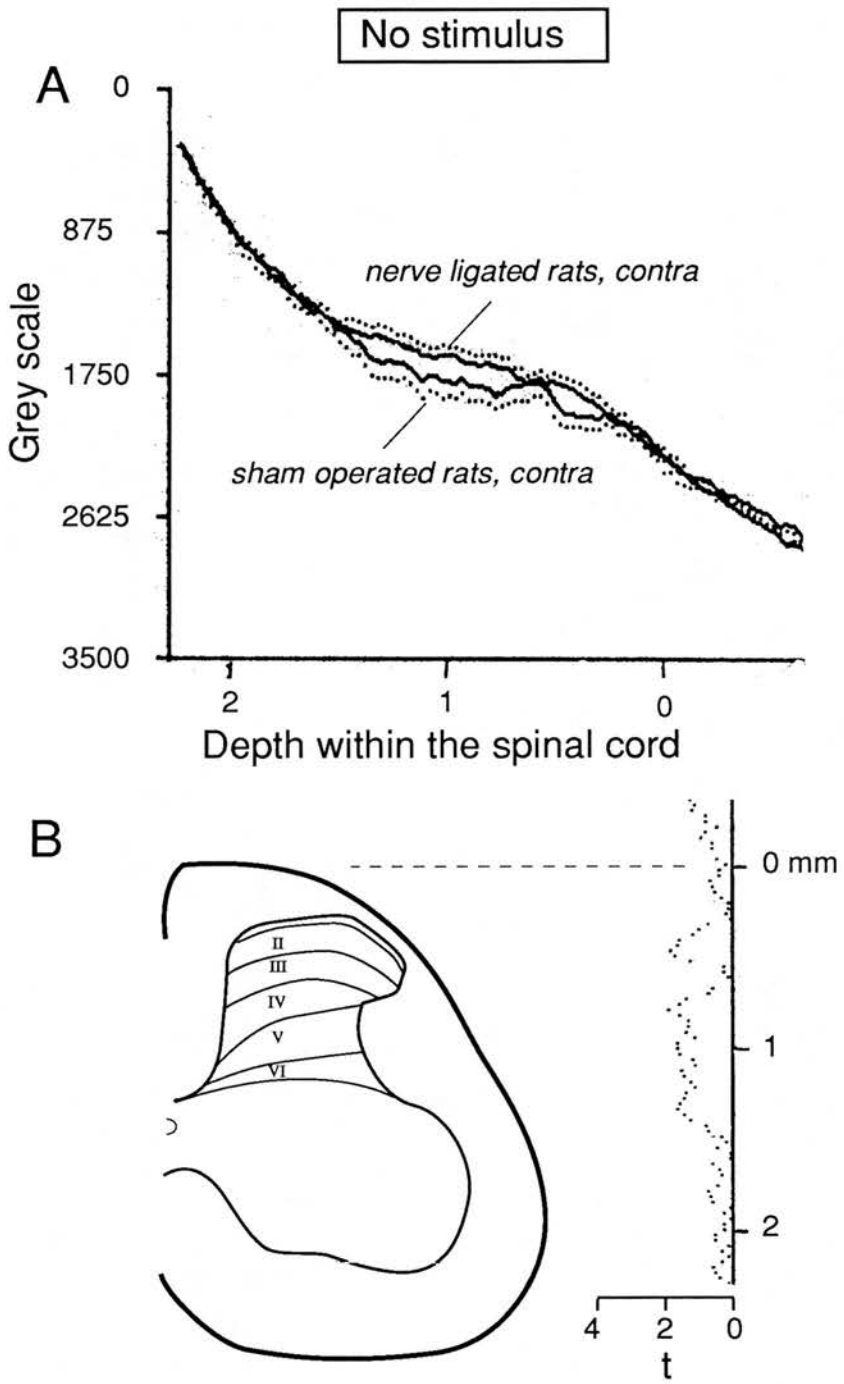

No stimulus
A
0
875
1750
2625
3500
Grey scale
nerve ligated rats, contra
sham operated rats, contra
2
1
0
Depth within the spinal cord
B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

Figure 19. Additional zone of release of ir-NPY in the ipsilateral spinal cord of nerve ligated rats when compared to the contralateral cord of sham operated rats.

(A) The mean image analysis of two groups of microprobes present in the spinal cord for 15 minutes in the absence of any active peripheral stimuli were plotted with respect to depth within the spinal cord: those present in the spinal cord ipsilateral to the nerve ligation (*nerve ligated rats, ipsi* n=53*)* and those present in the spinal cord ipsilateral to the sham operation (*sham operated rats, ipsi* n=19*)*. (B) The differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. The hatched area indicates where these differences are significant at the $P<0.05$ level.

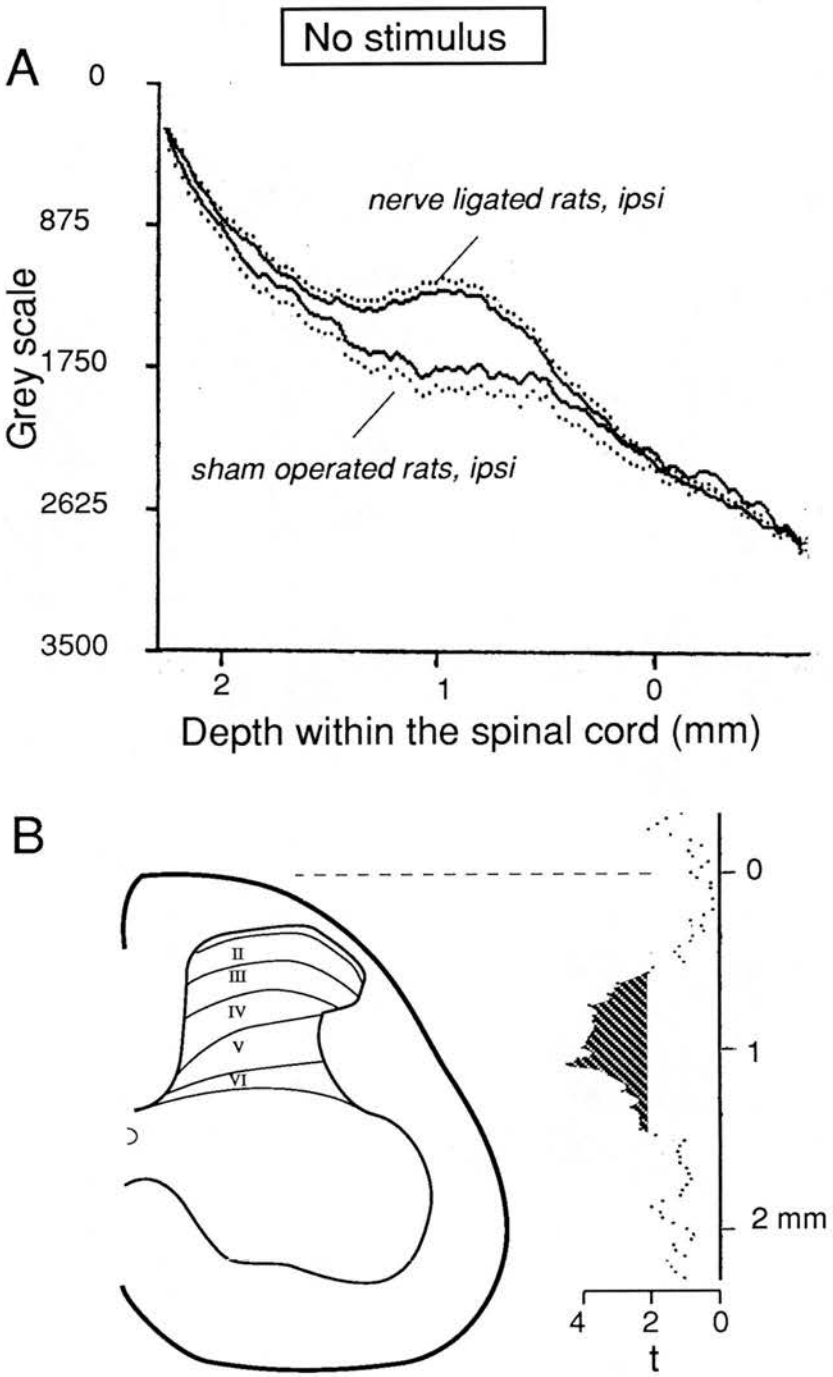
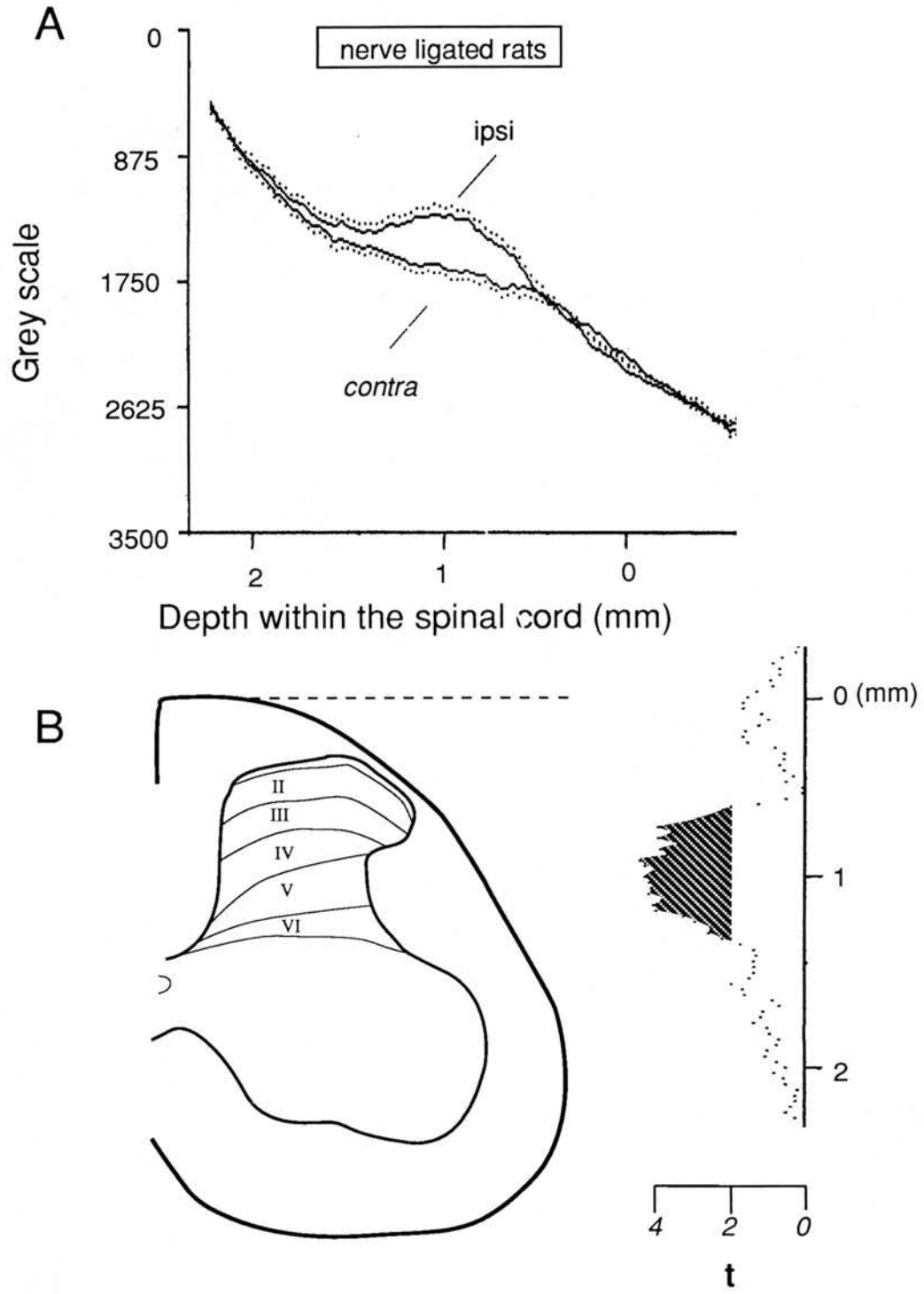

No stimulus
A
0
875
1750
2625
3500
Grey scale
nerve ligated rats, ipsi
sham operated rats, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0
1
2 mm
4
2
0
t

Figure 20. Additional zone of release of ir-NPY in the ipsilateral spinal cord of nerve ligated rats when compared to the opposite side of the spinal cord.

(A) The mean image analysis of two groups of microprobes present in the spinal cord for 15 minutes in the absence of any active peripheral stimuli were plotted with respect to depth within the spinal cord: those present in the spinal cord ipsilateral to the nerve ligation (*nerve ligated rats, ipsi* n=53) and those present in the opposite side of the spinal cord (*nerve ligated rats, contra* n=55). (B) The differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. The hatched areas indicates where these differences are significant at the $P<0.05$ level.


A
nerve ligated rats
ipsi
contra
Grey scale
0
875
1750
2625
3500
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

6.2.(e) Peripheral nerve stimulation and the spinal release of ir-NPY in nerve ligated rats

As shown earlier in this dissertation, electrical stimulation of the sciatic nerve in the normal rat failed to release ir-NPY. It was of interest to investigate whether electrical stimulation of the sciatic nerve proximal to the ligatures resulted in the release of ir-NPY in the nerve ligated animal as a *de novo* synthesis of NPY occurs in the medium to large sized dorsal root ganglia after nerve injury. Both large and small diameter fibres were stimulated at frequencies used previously: 20Hz (3 x T) to stimulate large myelinated fibres and 2Hz(>100 T) to activate both myelinated and unmyelinated fibres.

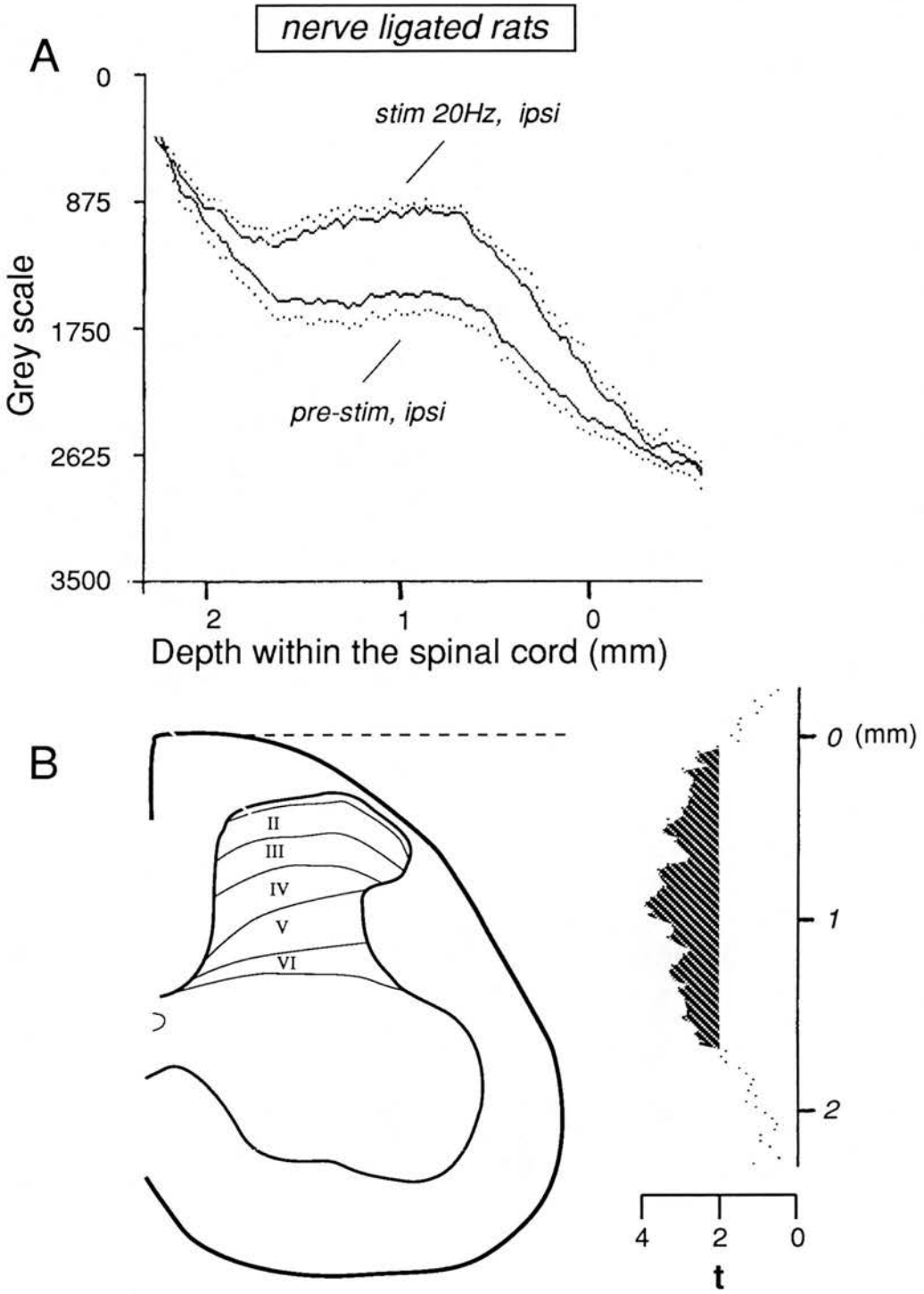
In all experiments only the injured nerve was stimulated. Bilateral changes in the excitatory postsynaptic effects of impulses after electrical stimulation of large fibres in the sciatic nerve of this model have been reported (Colvin et al, 1996). Therefore, it was also of interest to investigate whether ipsilateral nerve stimulation altered the basal presence of ir-NPY in the spinal cord contralateral as well as ipsilateral to the ligated nerve. Only one 15 minute period of stimulation was used in these experiments (similar to experiments in normal animals) due to the uncertainty of whether persistence of release of ir-NPY occurs following peripheral nerve stimulation. Four microprobes were inserted into the spinal cord prior to stimulation, two microprobes during electrical stimulation of the sciatic nerve and six after stimulation in either the cord ipsilateral or contralateral to the nerve injury.

6.2.(ei) Stimulation of large myelinated fibres at 20Hz

In 13 rats microprobes were inserted into the spinal cord before during and after one 15 minute period of electrical stimulation at 20Hz (3 x T). In figure 21A the mean image analyses of 14 microprobes present in the spinal cord ipsilateral to the injured nerve during stimulation of large diameter afferents at 20Hz is displaced above

Figure 21. Release of ir-NPY in the ipsilateral spinal cord of nerve ligated rats by large myelinated fibre stimulation of the sciatic nerve at 20Hz.

(A) The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in the spinal cord ipsilateral to the nerve ligation in the absence of peripheral stimuli (*pre-stim, ipsi* n=21) and those present in the same side of the cord during stimulation of large myelinated fibres at 20Hz (3 x T, 0.05ms, *stim 20Hz, ipsi* n=14). (B) A plot of the t-statistics is related to an outline of the lumbar spinal cord. Significant differences at the $P<0.05$ level are indicated by the hatched areas.


nerve ligated rats
A
0
875
1750
2625
3500
Grey scale
stim 20Hz, ipsi
pre-stim, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

those of 21 microprobes present in the same side before stimulation. This displacement is the entire length of the probe. Figure 21B shows that these injured nerve during stimulation of large diameter afferents at 20Hz is displaced above differences are significant throughout the dorsal white matter, the entire dorsal horn, dorsal columns and ventral horn. Microprobes were inserted into the spinal cord for a further three periods after electrical stimulation to determine whether persistence occurred following release.

These 3 post-stimulus intervals were grouped to give the mean image analysis of 32 microprobes inserted into the spinal cord from 5-60 minutes following stimulation. The mean image analyses of 21 microprobes inserted into the spinal cord ipsilateral to the nerve ligation in the absence of any active stimuli (before stimulation) is compared with that of 32 microprobes inserted into the ipsilateral spinal cord within the period 5-60 minutes after stimulation in figure 22A. This latter group is displaced above the pre-stimulus from approximately 1.8mm to the dorsal cord surface and suggests persitence of previously released ir-NPY. These differences, however, are not significant at the $P<0.05$ level although the t-values are close to 2 in the upper ventral horn. The time intervals were also analysed separately. The differences between the first period (inserted from 5-10 minutes after stimulation, n=9), second period (from 25-35 minutes, n=16) and the third post-stimulation period (from 45-60 minutes, n=7) mean image analysis groups when compared to the pre-stimulus group were not significant at the $P<0.05$ level (not illustrated).

To examine the possibility that stimulation above the nerve ligature was releasing ir-NPY bilaterally, microprobes were also inserted into the contralateral spinal cord during and after periods of nerve stimulation. In figure 23(i)A the mean image analysis of 9 microprobes inserted into the contralateral spinal cord during stimulation at 20Hz (*stim 20Hz, contra*) is displaced below the mean image analysis of 17 microprobes inserted into the same side of the cord before stimulation at 20Hz (of the ipsilateral nerve) which suggests inhibition of tonically released ir-NPY. The

Figure 22. Detection of ir-NPY in the ipsilateral spinal cord following sciatic nerve stimulus evoked release in nerve ligated rats.

(A) The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, ipsi* n=21*)* and 32 microprobes present in the spinal cord ipsilateral to the nerve ligation from 5-60 minutes after stimulation at 20Hz (3 x T, 0.05ms, *post-stim, ipsi*). (B) The differences between the two groups of microprobes are plotted at 30μm intervals with respect to an outline of the lumbar spinal cord. The absence of hatched areas indicates that these differences are not significant at the $P<0.05$ level although some of the t values are close to 2.

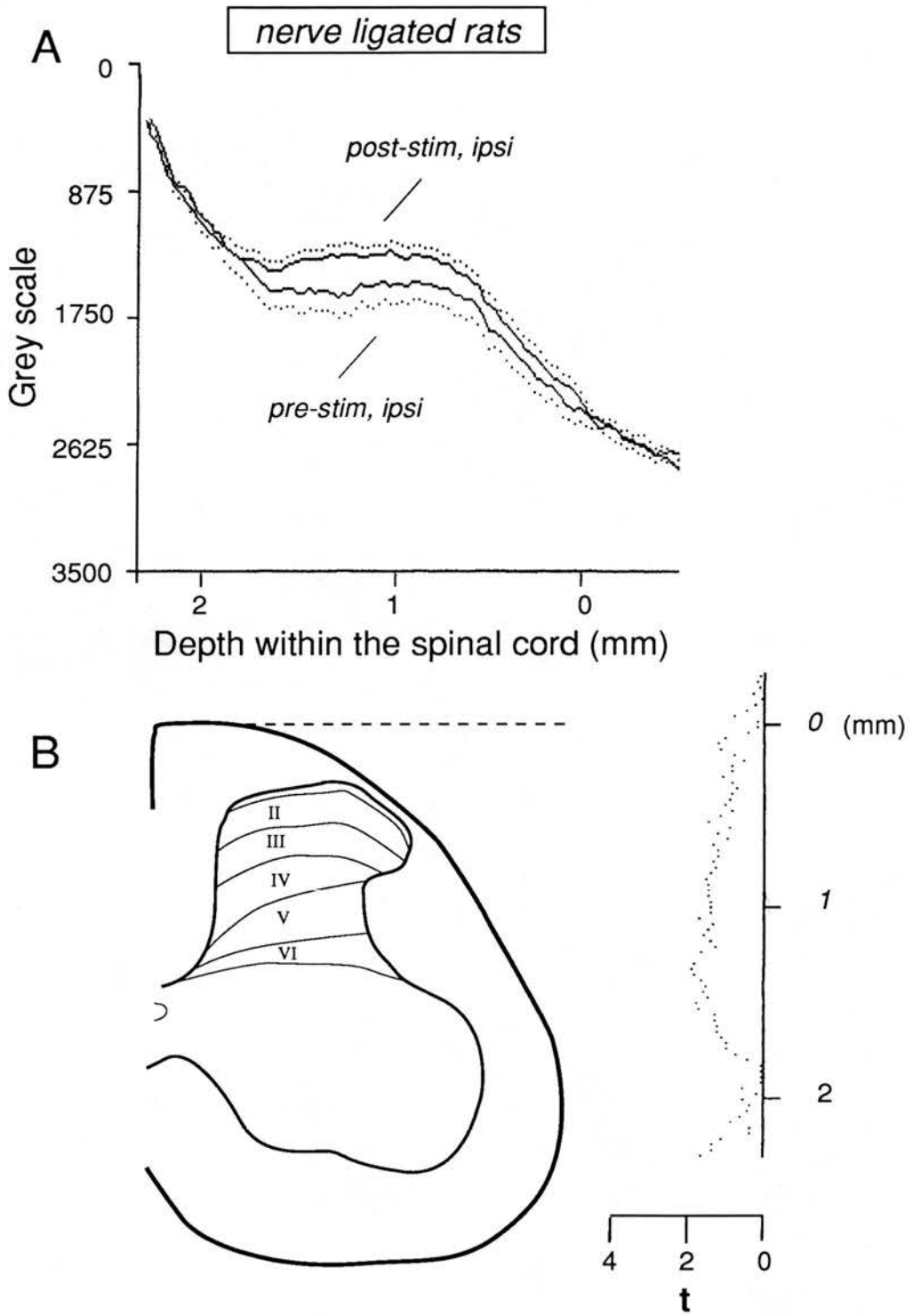
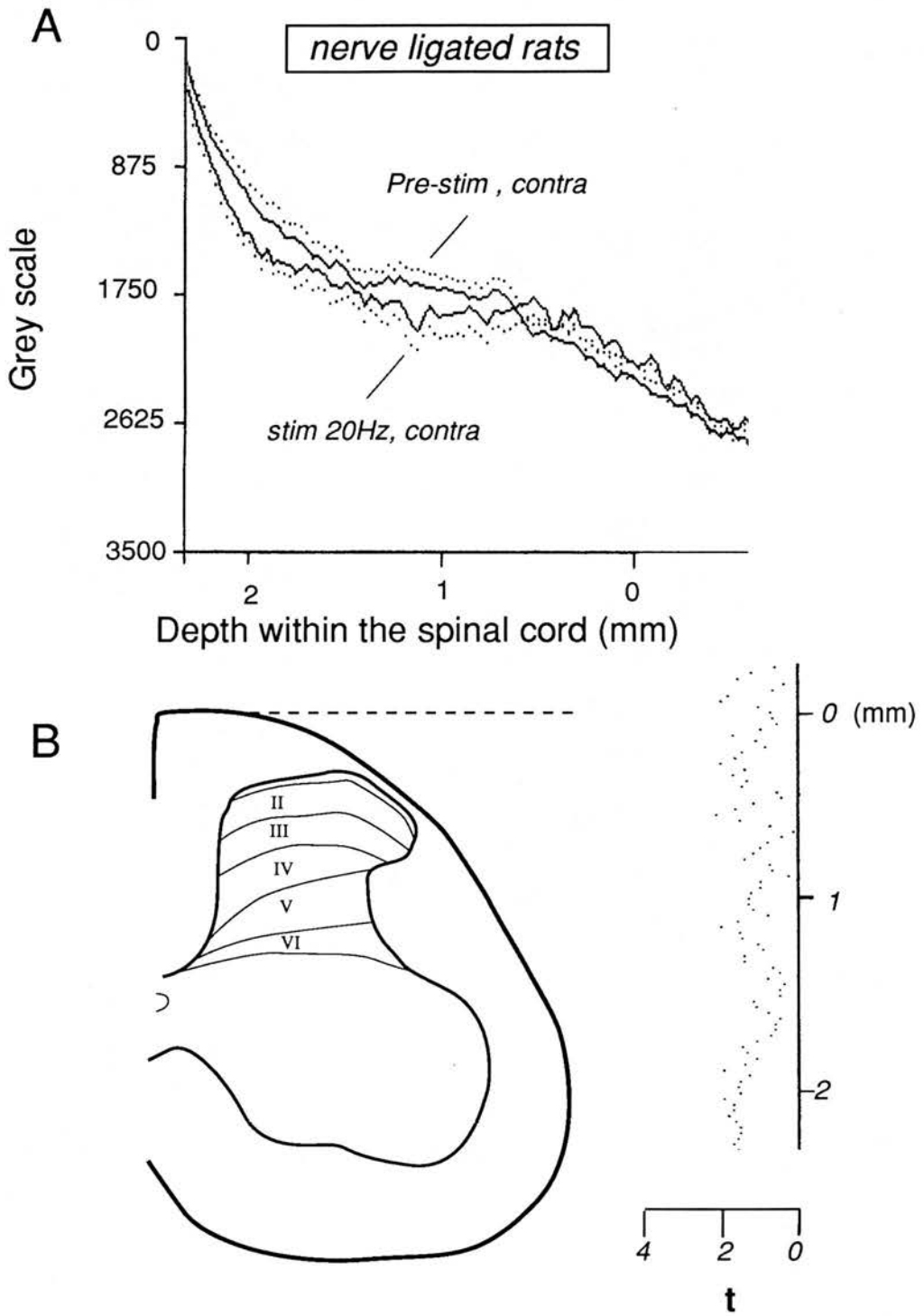

A
nerve ligated rats
0
875
1750
2625
3500
Grey scale
post-stim, ipsi
pre-stim, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

Figure 23. The effect of myelinated fibre stimulation of the sciatic nerve at 20Hz on ir-NPY in the contralateral spinal cord of nerve ligated rats

23(i) microprobes present during stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes were plotted with respect to depth within the spinal cord**:** 9 microprobes present in the spinal cord contralateral to the nerve during stimulation at 20Hz (3 x T, 0.05ms, *stim 20Hz, contra*) are displaced below those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, contra* n=17*)*. (B) The differences between the two groups of microprobes are plotted with respect to an outline of the lumbar spinal cord. Since only isolated points attain significance at the $P<0.05$ level there is no cross hatching.


A
nerve ligated rats
0
875
1750
2625
3500
Grey scale
Pre-stim , contra
stim 20Hz, contra
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

t-statistics illustrate that these differences are mainly not significant with only 4 separate and widely spaced 30μm intervals making significance (figure 23(i)B). The mean image analysis of microprobes inserted into the contralateral spinal cord after stimulation (from 5-65 minutes, n=35) is compared with the pre-stimulation plot in figure 23(ii)A. The differences are not significant at the $P<0.05$ level.

6.2.(eii) Stimulation of myelinated and unmyelinated fibres at 2Hz

Microprobes were inserted into the spinal cord before during and after one 15 minute period of electrical stimulation at 2Hz (>100 T) in 11 rats. In figure 24A the mean image analyses of 18 microprobes present in the spinal cord ipsilateral to the injured nerve before stimulation is compared to those present in the same side during stimulation at 2Hz (n=9). This latter group is displaced above the before-stimulation group along the entire length of the probe analysed. Figure 24B shows that these differences are significant throughout laminae I to IV and peaks at laminae III/IV.

The 3 post-stimulus intervals were grouped to give the mean image analysis of 27 microprobes inserted into the ipsilateral spinal cord from 5-50 minutes following stimulation. This plot is compared to the mean image analyses of 18 microprobes inserted into the ipsilateral spinal cord before stimulation in figure 25(i)A. The post-stimulus group is displaced above the pre-stimulus group from 2.25mm to the dorsal cord surface. The differences are significant in part of laminae III-IV and laminae V,VI and upper laminae VII.

The 3 post-stimulus time intervals were also analysed separately and are illustrated in figure 25(ii)A, B, C (all n=9). At all three intervals, the mean image analysis plot of microprobes inserted into the ipsilateral spinal cord after stimulation at 2Hz is displaced above the mean image analysis of microprobes inserted into the spinal cord prior to stimulation. These results suggests that the released ir-NPY persisted after stimulation. The displacement above the no stimulus microprobe plot

Figure 23. The effect of myelinated fibre stimulation of the sciatic nerve at 20Hz on ir-NPY in the contralateral spinal cord of nerve ligated rats

23(ii) microprobes present after stimulation.

The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes in the absence of any active peripheral stimuli are plotted: those present in both sides of the spinal cord prior to stimulation at 20Hz (*pre-stim, contra* n=17) and those present in the ipsilateral spinal cord from 5-65 minutes following stimulation (*post-stim, contra* n=35). The two groups are not significantly different at the $P<0.05$ level.

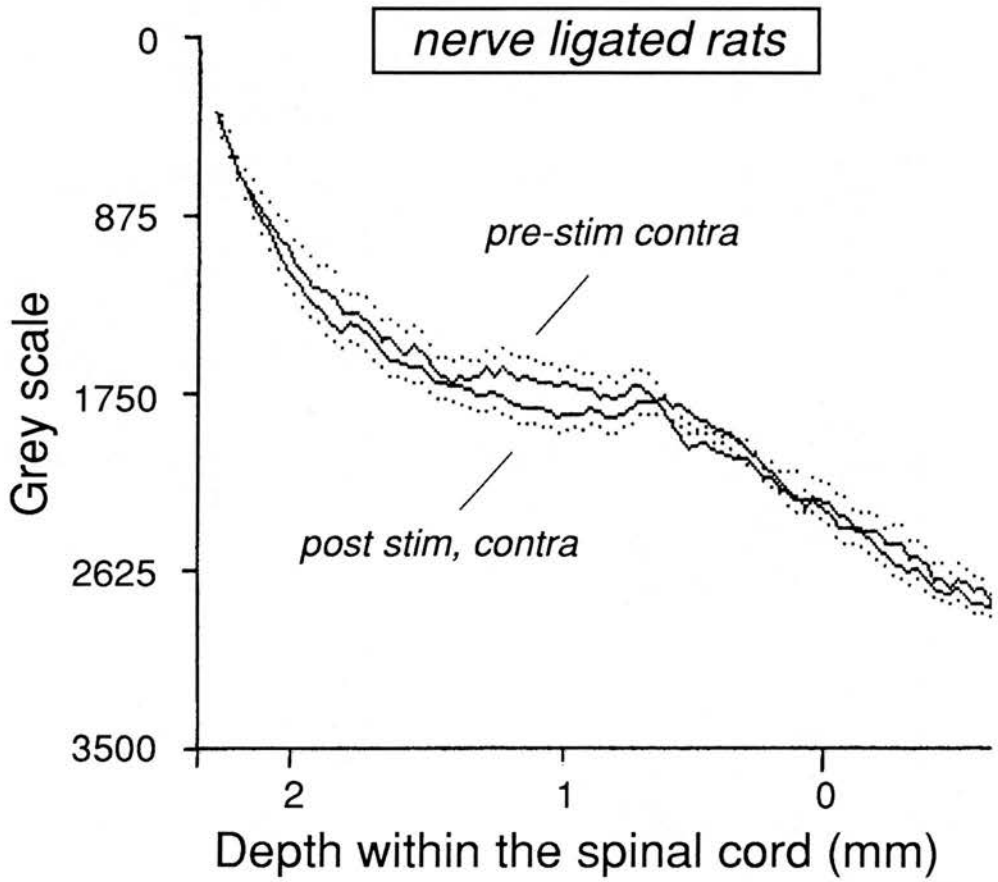

nerve ligated rats
0
875
1750
2625
3500
Grey scale
pre-stim contra
post stim, contra
2
1
0
Depth within the spinal cord (mm)

Figure 24. Release of ir-NPY in the ipsilateral spinal cord of nerve ligated rats during stimulation of large myelinated fibre and unmyelinated fibres of the sciatic nerve at 2Hz.

(A) The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, ipsi* n=18) and 9 microprobes present in the spinal cord ipsilateral to the nerve ligation during stimulation at 2Hz (>100 x T, 0.05ms, *stim 2Hz, ipsi*). (B) A plot of the t-statistics at each 30μm interval is related to an outline of the lumbar spinal cord. The hatched area indicates where these sites are significant at the $P<0.05$ level.

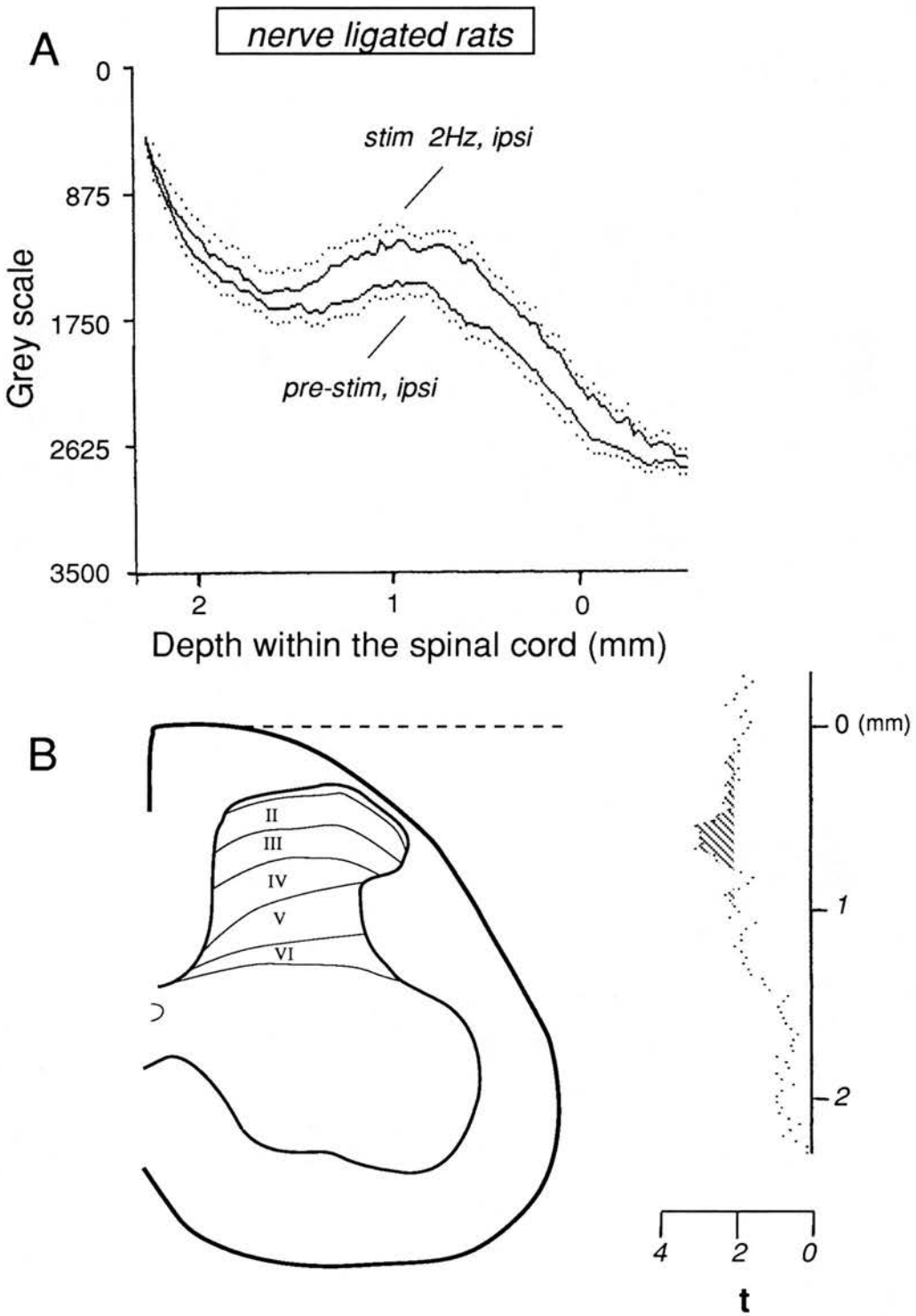

A
nerve ligated rats
0
875
1750
2625
3500
Grey scale
stim 2Hz, ipsi
pre-stim, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

Figure 25. Persistence of release of ir-NPY in the ipsilateral spinal cord of nerve ligated rats after stimulation of large myelinated and unmyelinated fibres of the sciatic nerve at 2Hz.

(i) microprobes inserted 5-50mins following stimulation

(A) The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, ipsi* n=18) and 27 microprobes present in the spinal cord ipsilateral to the nerve ligation from 5-50 minutes after stimulation at 2Hz (>100 x T, 0.05ms, *post-stim 2Hz, ipsi*). (B) The differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. Sites of significance at the $P<0.05$ level are indicated by the hatched area.

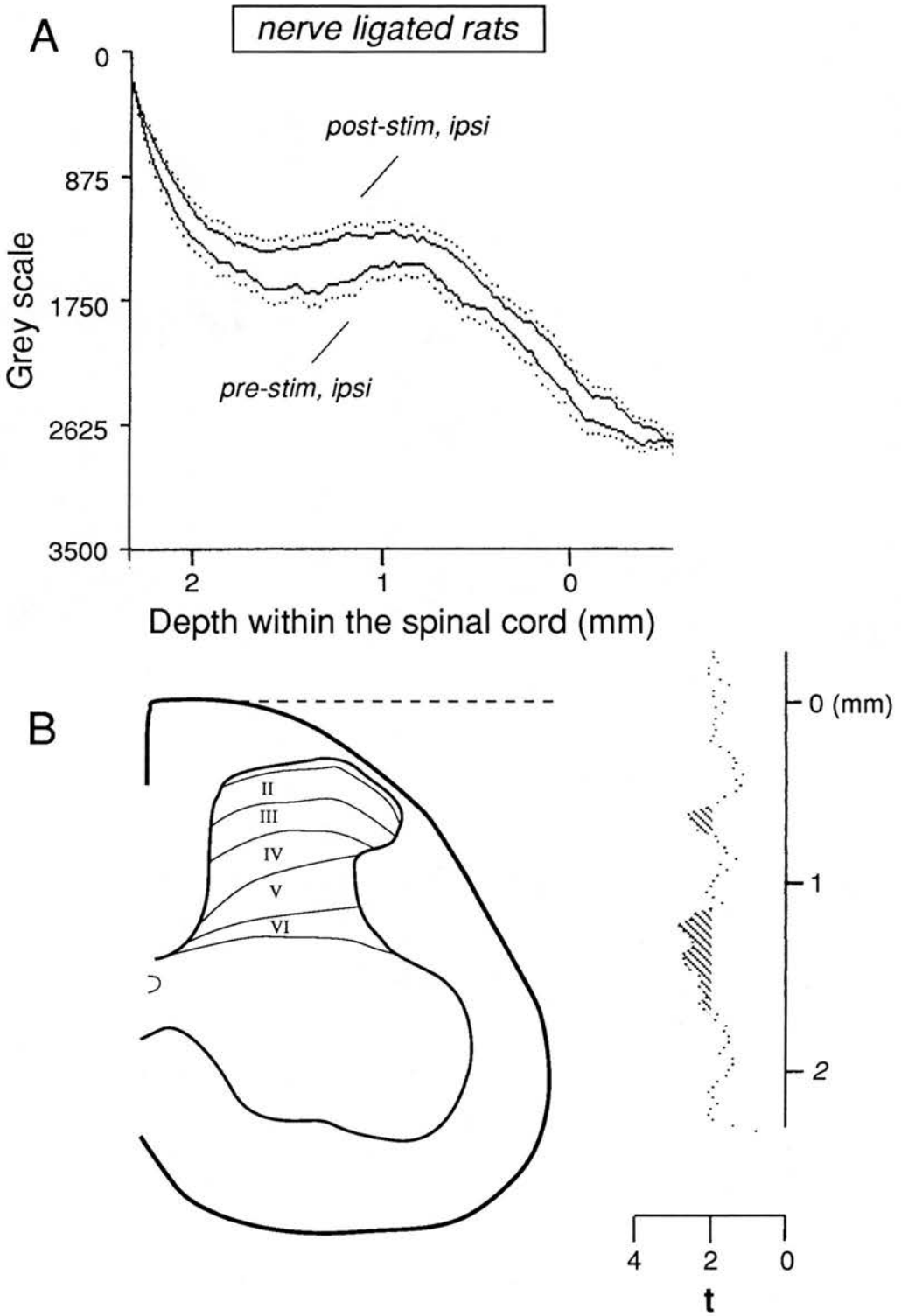
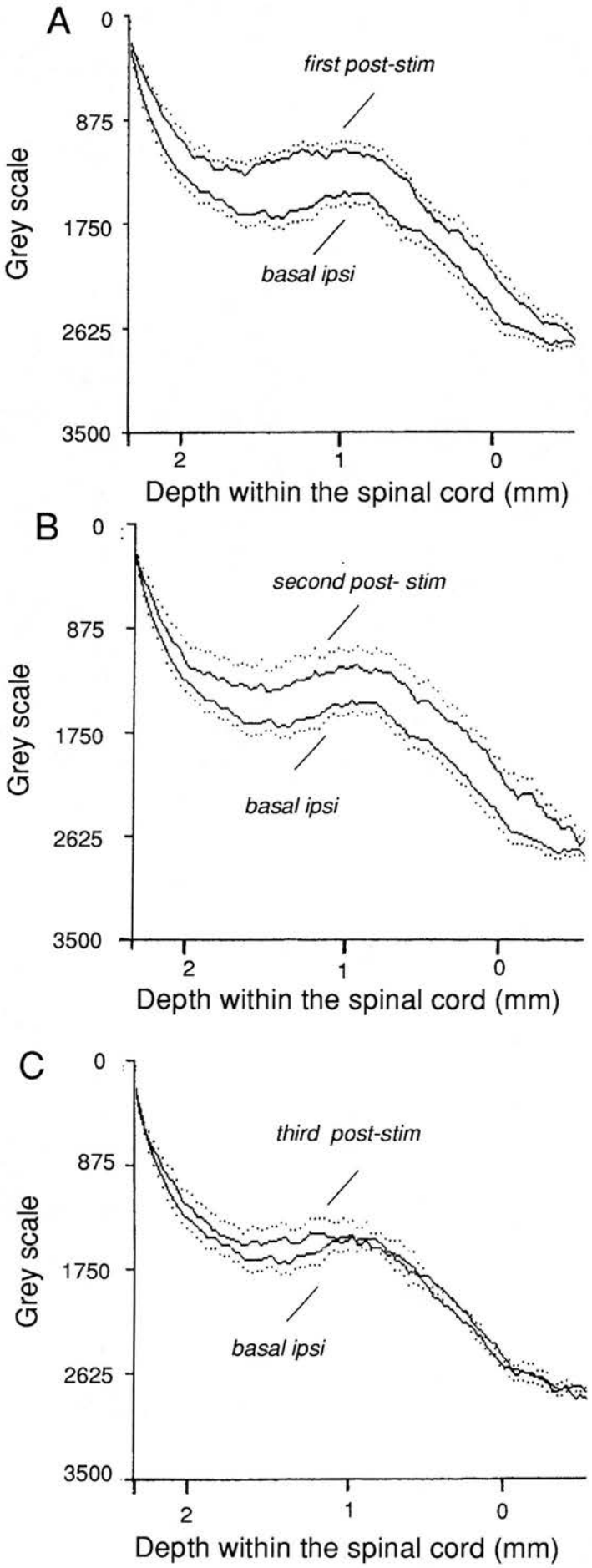

A
nerve ligated rats
0
875
1750
2625
3500
Grey scale
post-stim, ipsi
pre-stim, ipsi
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 (mm)
1
2
4
2
0
t

Figure 25. Persistence of release of ir-NPY in the ipsilateral spinal cord of nerve ligated rats after stimulation of large myelinated and unmyelinated fibres of the sciatic nerve at 2Hz.

(ii) microprobes inserted 5-10mins, 25-30mins & 40-50 mins following stimulation

The mean image analysis of microprobes present in the spinal cord for 15 minutes in both sides of the spinal cord of nerve ligated rats in the absence of any active peripheral stimuli (*pre-stim, ipsi* n=21*)* is compared with (A) 9 microprobes present in the spinal cord ipsilateral to the nerve ligation 5-10 minutes after stimulation at 2Hz (*first post-stim, ipsi),* (B) 9 microprobes present in the spinal cord ipsilateral to the nerve ligation 25-30 minutes after stimulation (*second post-stim, ipsi*) and (C) 9 microprobes present in the spinal cord ipsilateral to the nerve ligation 40-50 minutes after stimulation (*third post-stim, ipsi*). The first post-stimulation plot differs from the pre-stimulation plot in the lower dorsal and upper ventral horn. The second post-stimulation plot differs from the pre-stimulation plot at only a few sites in the dorsal horn whereas the third post-stimulation plot is not significantly different from the pre-stimulation group.


A
0
875
1750
2625
3500
Grey scale
first post-stim
basal ipsi
2
1
0
Depth within the spinal cord (mm)
B
0
875
1750
2625
3500
Grey scale
second post- stim
basal ipsi
2
1
0
Depth within the spinal cord (mm)
C
0
875
1750
2625
3500
Grey scale
third post-stim
basal ipsi
2
1
0
Depth within the spinal cord (mm)

decreases with time suggesting a progressive degradation of released ir-NPY. In figure 25(ii)A the post stimulation plot of microprobes inserted into the spinal cord from 5-10 minutes after stimulation is significantly different in laminae II-VI when the differences between this group and the basal group are compared. The second post-stimulation mean image analysis of microprobes inserted into the spinal cord 25-30 minutes after stimulation is significantly different from the basal plot at only a few sites in the dorsal horn. In contrast, the differences between the third post-stimulation plot of microprobes inserted from 40-50 minutes after stimulation are not significant at the $P<0.05$ level.

The possibility of bilateral nerve stimulus-evoked release was examined. In figure 26(i) the mean image analysis of 12 microprobes inserted into the contralateral spinal cord during stimulation at 2Hz is very similar to the mean image analysis of 19 microprobes inserted into the same side of the cord prior to nerve stimulation at 2Hz and the mean image analysis of microprobes inserted into the contralateral spinal cord from 5-55 minutes after stimulation (n=27, figure 26(ii)). The differences between these groups are not significant which suggests that electrical stimulation of the ipsilateral nerve at 2Hz did not result in bilateral release.

Figure 26. Failure of stimulation of large myelinated fibres and unmyelinated fibres of the sciatic nerve at 2Hz to release ir-NPY contralateral to the nerve ligation.

26 (i) microprobes present during stimulation

The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, contra* n=19*)* and 12 microprobes present in the spinal cord ipsilateral to the nerve ligation during stimulation at 2Hz (>100 x T, 0.05ms, *post-stim 2Hz, contra*). A plot of the t-statistics derived from the standard errors of the differences of means at each 30μm interval is related to an outline of the lumbar spinal cord. There are no significant differences between the two groups.

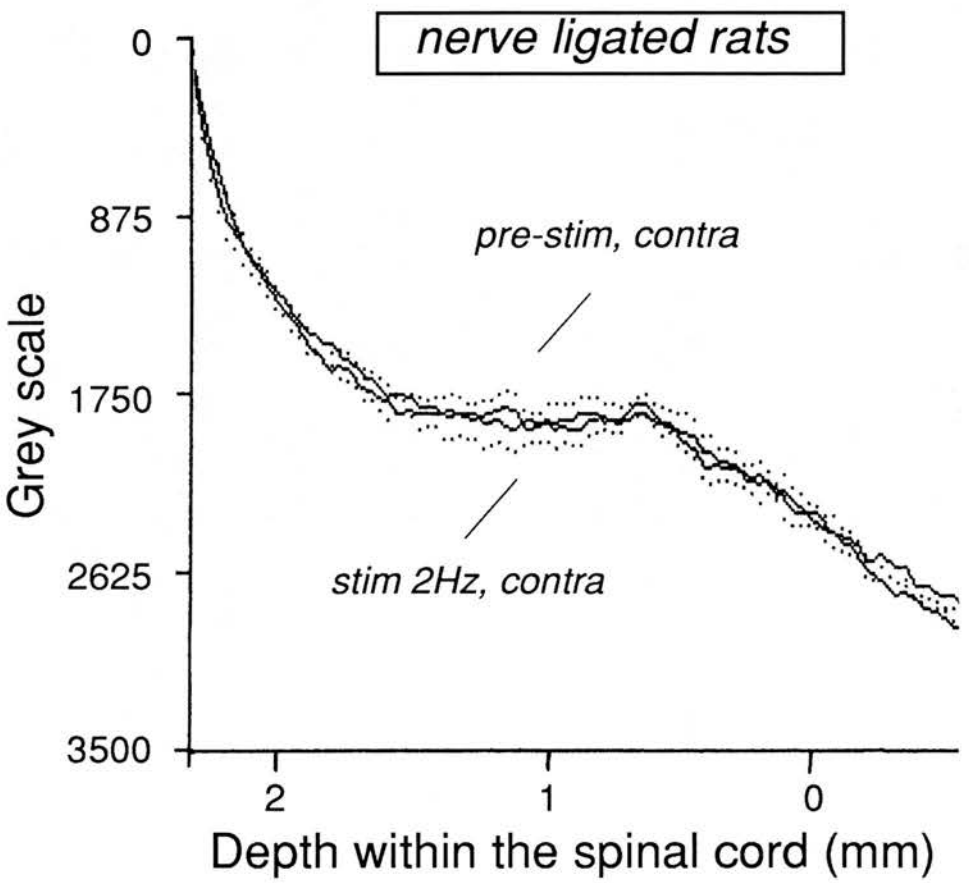
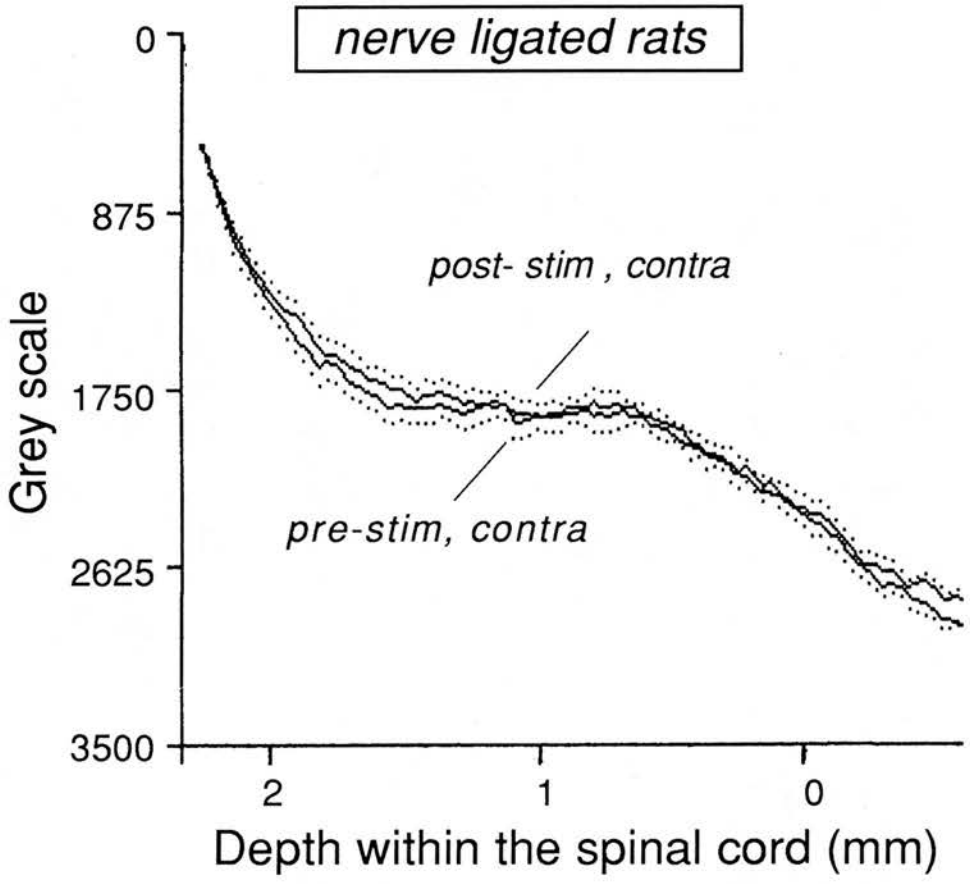

nerve ligated rats
0
875
1750
2625
3500
Grey scale
pre-stim, contra
stim 2Hz, contra
2
1
0
Depth within the spinal cord (mm)

Figure 26. Failure of stimulation of large myelinated fibres and unmyelinated fibres of the sciatic nerve at 2Hz to release ir-NPY contralateral to the nerve ligation

26 (ii) microprobes present after stimulation

The mean image analysis of two groups of microprobes present in the spinal cord of nerve ligated rats for 15 minutes were plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord in the absence of any active peripheral stimuli (*pre-stim, contra* n=19) and 27 microprobes present in the spinal cord contralateral to the nerve ligation from 5-55 minutes after stimulation at 2Hz (*post-stim, contra*). These differences are not significant at the $P<0.05$ level.


nerve ligated rats
0
875
1750
2625
3500
Grey scale
post- stim , contra
pre-stim, contra
2
1
0
Depth within the spinal cord (mm)

6.3. DISCUSSION

The significant results of this study which require extended discussion are:

(a) the finding of a comparable basal extracellular presence of ir-NPY in both sides of the spinal cord in sham operated animals and in the side contralateral to the ligated nerve.

(b) the deep zone of basal release of ir-NPY in the ipsilateral spinal cord of nerve ligated rats which was not present in sham operated rats nor the contralateral spinal cord.

(c) the release of ir-NPY by electrical stimulation of primary afferents in nerve ligated but not normal rats.

6.3.(a) The basal presence of ir-NPY in the spinal cord of the sham and nerve ligated rat

Sham experiments were carried out in separate animals to allow both sides of the spinal cord to be investigated after a unilateral sham operation or a unilateral sciatic nerve ligation. A basal presence of ir-NPY in sham operated animals was found throughout the dorsal horn and was similar for both sides of the spinal cord. This basal presence can also be called basal release as it is most likely due to continuous activity in some structures under the conditions of these experiments since it was obtained in the absence of any active peripheral stimuli. Basal release in sham operated rats was comparable to that of normal rats. The spinal cord contralateral to the ligatured nerve also had a comparable distribution of extracellular ir-NPY to that detected in the sham operated animals.

In contrast, significant differences from this distribution were obtained for the ipsilateral side of nerve ligated rats . In addition to release in the upper and mid-dorsal horn (which was comparable to normal animals), there was a new zone of

release of ir-NPY in the mid and deep dorsal horn (approximating to laminae IV, V & VI). The latter was observed in the absence of any active peripheral stimuli. Determining the sources of such release requires consideration of other results which I obtained. The first of these is the effects of nerve stimulation.

6.3.(b) Release by electrical stimulation of the ligated sciatic nerve

Electrical stimulation of large myelinated fibres and of myelinated and unmyelinated fibres elicited release in the ipsilateral side of the spinal cord in nerve ligated animals whereas the same nerve stimulation did not elicit release in normal rats. Electrical stimulation of these fibres did not alter the spatial distribution of extracellular ir-NPY but simply elevated the levels present prior to electrical stimulation in the dorsal horn and upper ventral horn. This result implies that spontaneous activity in large diameter primary afferents of the ligatured nerve could explain the basal pattern of release seen in the ipsilateral spinal cord.

The distribution of release after stimulation at 20Hz (3 x T) was more extensive than that obtained at 2Hz (>100 T) which may simply be related to frequency dependence as large myelinated fibres would have been stimulated by both frequencies. The low frequency was used with high intensity stimuli in these experiments to activate C fibres. Alternatively, electrical stimulation at 2 Hz (>100 T) may result in the activation of an inhibitory process within the spinal cord resulting in release over a restricted area.

6.3.(c) What is the origin of the deep zone of basal release in nerve ligated rats?

After axotomy or nerve constriction a *de novo* synthesis of NPY has been reported to occur predominantly in a population of large to medium sized dorsal root

ganglion neurones (Wakisaka et al, 1992; Zhang et al, 1993a & b; Kashiba et al, 1994, Nahin et al, 1994). This correlates with the finding that electrical stimulation of the low threshold (large diameter) fibres proximal to the constriction site was adequate to elicit release of ir-NPY although it failed to elicit release in normal rats. Zhang and co-workers found that many synaptic vesicles in laminae III and IV were present in the presynaptic zone and many vesicles appeared to be involved in exocytosis which suggests increased transmitter release (Zhang et al, 1995b).

If the phenotypes of large neurones synthesising NPY following peripheral axon damage includes both proprioceptive and large fibre mechanoreceptors then impulses in these fibres would result in release of NPY in mid and lower dorsal horn and the upper ventral horn as found in the present experiments.

Attempts by the nerve to regenerate after peripheral nerve injury may be similar to the dependence of primary afferent neurones on neurotrophins during development. Local application of NT-3 directly to the proximal transected nerve has been found to decrease the number and intensity of staining of ir-NPY on the ipsilateral side in the dorsal root ganglion neurones and in their central terminals in the dorsal horn (Ohara et al, 1995). This study suggested that the synthesis of NPY is a response to the deprivation of retrogradely transported NT-3. Mice deficient in the gene for synthesis of NT-3 have been found to have movement deficits associated with abnormal limb positions. These mice have an extensive loss of large diameter dorsal root ganglion neurones and afferent fibres projecting to the deep dorsal and upper ventral horn are absent (Farinas et al, 1994; Ernfors et al, 1994; Airakinsen & Meyer 1996). Both Ia & Ib muscle afferents which project to this area of the cord and their peripheral sense organs, muscle spindles and Golgi tendon organs have been found to be absent (Ernfors et al, 1994). In view of the severe gait abnormalities which these animals have, it has been proposed that the development of proprioceptive (muscle spindle and tendon organ derived) afferents is NT-3 dependent. Another study found that the Merkel cells of the skin, the end organs of

slowly adapting mechanoreceptors decreased after birth in NT-3 deficient mice suggesting that the development of these cells is NT-3 dependent (Airakinsen et al, 1996). These afferents project to laminae III/IV in normal animals (Brown 1981).

The effect of NT-3 on the regulation of NPY is likely to be mediated by tyrosine kinase C (TrkC) receptors which are predominantly expressed in large-sized dorsal root cells subserving proprioceptive functions (Ip et al, 1992; Mu et al, 1993; McMahon & Priesley 1995). Mice with a knockout gene for the TrkC receptor were found to have movement disorders and a guarded foot. Again a reduction in the large dorsal root ganglia expressing this receptor was found as well as a reduction in the number of Ia afferents (Klein et al, 1994). In contrast to the study by Airakinsen and co-workers, Klein's group suggested that projections to the dorsal horn of low threshold mechanoreceptors were grossly normal. This may be due to the earlier time course studied by Klein and co-workers.

Immunocytochemical studies of ir-NPY distribution in the dorsal horn following sciatic nerve transection (Wakisaka et al, 1991b; Kashiba et al, 1994; Zhang et al, 1995b) and partial injury (Wakisaka et al, 1992; Munglani et al, 1995) have found an increase in lamina III -V of the dorsal horn. Munglani and co-workers found that pre-emptive treatment with MK-801 reduced the magnitude of the increase in NPY within structure in laminae III & IV (Munglani et al, 1995). They suggested that MK-801 may have reduced the effect of the injury discharge within the spinal cord. Lumbar dorsal rhizotomy performed simultaneously with sciatic nerve transection prevented the development of NPY immunoreactivity in laminae III & IV suggesting that the NPY was contained within primary afferent fibres (Ohara et al, 1994). This distribution is consistent with the termination of slowly adapting mechanoreceptors but not with the presence within muscle spindle derived afferents as these terminate more deeply in normal rats. Thus, the increased synthesis of NPY may not be wholly NT-3 dependent. The actions of nerve growth factor (NGF) in regulating peptide expression have been examined *in vivo* in adult rat primary sensory

neurones (Verge et al, 1995). Delayed intrathecal infusion of NGF for 7 days starting 2 weeks after injury reduced the number of neurones expressing VIP, cholecystokinin, NPY and galanin after injury. NGF appears to regulate peptide expression differentially and may also be part of the signal that allows reversion to normal of responses to injury as axons regenerate.

Thus it appears likely that the source of this extracellular release of ir-NPY arises from the large fibres which synthesise this peptide after injury as: (1) electrical stimulation of large myelinated fibres results in release in neuropathic rats contrasting to the failure to evoke release in normal rats and (2) the release of ir-NPY detected by the microprobes agrees with the distribution of central terminals of these large fibres and increased immunoreactivity after injury.

6.3.(d) The stimulus for release in neuropathic animals

As mentioned previously NPY release in the ipsilateral spinal cord of rats with a nerve ligature rats is found in the absence of any active peripheral stimuli. A possible source of this spontaneous activity is ectopic firing known to occur after partial and complete nerve injury. Ectopic impulses have been found to originate at the site of injury and/or in the dorsal root ganglion neurones in this model (Kajander & Bennett 1992, Study & Kral 1996, Tal & Eliav 1996). This supports the proposal that the spontaneous release of ir-NPY originated from large afferent fibres as ectopic impulses have been found to continue for several weeks after partial nerve injury (Xie & Xiao 1990; Eliav & Tal 1994; Xie et al, 1995). It has been suggested that peripheral NPY may be involved in ectopic electrical activity in experimental neuromas (Fried et al, 1989). Ectopic impulses are normally regarded as pathological. However such impulses may be required after axonal damage to release the newly synthesised neuroactive compounds during the regenerative phase. Neuropeptide Y may be one such compound.

6.3.(e) The wide distribution of release

Where a neuropeptide is found following release however is not necessarily coincident with sites of release as considerable diffusion can occur following release if the compound is slowly degraded as has been reported with NKA (Nyberg et al, 1984; Hooper et al, 1985; 1987; Theodorsan-Norheim et al, 1987).

In the present studies the increase in extracellular ir-NPY evoked by nerve stimulation was suggested after stimulation at 20Hz (although this was not significant) and found to persist for up to 30 minutes after release following stimulation at 2Hz. Immunoreactive NPY was significantly elevated compared to basal levels 20-25 minutes after stimulation at 2Hz. By 40-50 minutes after stimulation, the next time period studied, the levels of ir-NPY had decreased. This suggests a progressive but slow degradation of released ir-NPY.

Little is known of how NPY is degraded following release in the central nervous system. With cultured cortical neurones, the degradation of NPY by plasmin (a serine proteinase) and plasminogen activator has been found to be slow (Ludwig et al, 1996). The post-stimulus results obtained with the antibody microprobes also suggest a slow degradation of released NPY. There is evidence with other neuropeptides that slow degradation results in considerable diffusion away from sites of release. Neuronal uptake into cells, which would limit the migration within the spinal cord tissue, has not been found for peptides. The persistence of NKA which is highly resistant to enzymatic degradation has been found to have a comparable time course to that of NPY in the present experiments as measured by the antibody microprobe technique (Hope et al, 1990b) and after superfusion of the cord dorsum (Beck et al, 1995). NKA has been found to diffuse widely in the spinal cord after release (Hope et al, 1990 b). Thus it is possible that the new zone of extracellular presence of ir-NPY is more extensive than the area of releasing neurones. The presence in the deep dorsal horn could have resulted from release more dorsally.

Supporting this is the extensive presence in the dorsal columns which almost certainly resulted from diffusion from the adjacent grey matter. Alternatively, these results could suggest that release continued after stimulation had ceased perhaps due to the altered properties of afferents in the nerve ligated animals. However, evidence to support this in other systems is lacking.

6.3.(f) Volume transmission

It has been suggested that long term changes in the CNS are mediated by extrasynaptic spread of neuropeptides as a mismatch of receptors and terminals for ir-NPY have been found in the brain (Agnati et al, 1989; Zoli et al, 1989). Fuxe and co-workers proposed the term volume transmission for such a process and much of the evidence was obtained from NPY's receptor distribution (Fuxe et al, 1990, Fuxe & Agnati 1991). Low affinity receptors (Y_1) were located adjacent to sites of release where high concentrations of the non-degraded peptide were encountered. In contrast, the high affinity receptors (Y_2) occurred more remotely. This latter receptor type was activated by both the intact peptide and shorter fragments degraded by peptidases whereas the low affinity receptor was only activated by the full length sequence of NPY.

They suggested that the vast majority of synapses operate not only via "hard wired" transmission (the classical system of chemical communication between cells which had been characterised on the basis of the minimal distance between release sites and target cells) but also by volume transmission. When the nerve cell is in low activity, wire transmission is the main mode in operation, whereas in high activity states, volume transmission becomes more important.

A marked plasticity in NPY receptor Y1 mRNA expression has been found 14 days after sciatic nerve transection (Zhang et al, 1994b). Before surgery, no large or medium sized dorsal root ganglion neurones of the lumbar region were found to

express this receptor compared to 60% after axotomy. Correlation studies by Zhang and co-workers indicated that NPY receptor mRNA & NPY mRNA were frequently co-localised after axotomy in large and medium dorsal root ganglion neurones (Zhang et al, 1994b). In contrast a down-regulation in small dorsal root ganglion neurones has been reported although a transient increase in the number of neurones expressing NPY receptor Y1 mRNA at low levels occurred. NPY released in the spinal cord may act upon receptors in the spinal cord. Y1 receptor RNA-positive neurones have been found in lamina II of the dorsal horn (Zhang et al, 1994b).

6.3.(g) Is NPY associated with the primary afferent fibres which sprout after nerve injury?

The growth associated protein GAP-43 has been found to be associated with axonal growth (Skene & Willard 1981). It has been shown to be increased in small & large dorsal root ganglion cells after peripheral nerve injury (Hoffman 1989; Sommervaille et al, 1991). Following nerve injury, ir-GAP-43 has been found in the damaged peripheral axons (Tetzlaff et al, 1989) and also at the central terminals of these afferents in the superficial dorsal horn of the spinal cord (Woolf et al, 1990; Coggeshall et al, 1991; Cameron et al, 1991). The majority of axons that show GAP-43 immunoreactivity have been found to be unmyelinated (Coggeshall et al, 1991) although some myelinated axons were also labelled at a later period (Somervaille et al, 1991). Woolf and co-workers (1990) suggested that the increase in GAP-43 production following sciatic nerve transection is due to inhibition of retrograde transport of a molecular signal from the periphery to neurones of the dorsal root ganglia. Peripheral axonal growth ensues following nerve injury but Woolf et al suggested that GAP-43 may permit central regenerative changes to occur. Such axonal sprouting may occur enabling the terminals of viable neurones to occupy synaptic sites which become vacant due to death of central terminations of neurones

as a result of the peripheral axotomy. This form of regeneration has been described as conditioned collateral sprouting (reviewed by Coggeshall, 1994).
Conditioned because it is elicited by injury to the peripheral process of the sensory neurone and collateral because the growth of terminals is from an uninjured neurone into a denervated region. An important question arising from these studies which need to be answered is whether these sprouting axons contain and release NPY.

Cholera toxin (B-fragments) conjugated to horseradish peroxidase (B-HRP) has been found to label myelinated sensory axons which have been reported to spread into lamina II after axotomy and partial nerve injury (Lekan et al, 1996). There is an increase in B-HRP labelled synapses into lamina II which have been suggested to arise from these sprouting A fibres (Woolf et al, 1992; Shortland & Woolf 1993; Koerber et al, 1994). The cells in lamina II normally have a predominantly monosynaptic input from unmyelinated nociceptors but if the sprouting fibres establish functional contacts inappropriate responses to innocuous stimuli could occur (Woolf et al, 1992). If these fibres which sprouted contained ir-NPY then increased staining in laminae I & II would have been expected after nerve injury. As ir-NPY has been reported to increase in laminae III & IV only, these fibres may be a subset of large fibres which do not sprout after peripheral nerve injury.

6.3.(h) Functional implications

Synthesised NPY in dorsal root ganglion cells may be transported both centrally and peripherally (Hisanaga et al 1993; Rao et al, 1992). Peripherally, NPY may induce the proliferation of Schwann cells and promote axonal regeneration (Shigeri & Fugimoto 1993). Centrally, it may induce a trophic effect on glial cells as does VIP (Brennenman et al, 1987; Hisanaga et al, 1993). The relative ease with which spontaneous release of ir-NPY was detected in the present experiments suggests an important role for NPY in the spinal cord response to peripheral nerve

injury. However, this role remains uncertain. Munglani and co-workers found a significant correlation between the increase in NPY staining and the degree of mechanical hyperalgesia and suggested that the increased expression of NPY centrally may be crucial for the development of hyperalgesia in this model (Munglani et al, 1995). They proposed that NPY may act to effectively silence transmission of impulses from primary afferents (Munglani et al, 1996b). As cited previously, NPY has been shown by a variety of techniques to inhibit the release of neurotransmitters (Walker et al, 1988; Bleakman et al, 1991) including that of substance P in the spinal cord (Duggan et al, 1991a). Intrathecal NPY has been found to have an antinociceptive action in the spinal cord which was enhanced after peripheral nerve transection (Xu et al, 1994). However, the excitatory effect of NPY (as it is usually biphasic in normal animals), was also increased. A later study by Munglani and co-workers reported that the resolution of the hyperalgesia occurs before the central nerve changes have been resolved (Munglani et al, 1996a). They suggested that long-term changes in neuropeptides within the central axons of injured nerves (particularly NPY) may serve to mask a residual hyperalgesia after a peripheral nerve injury. The injury itself despite apparent peripheral resolution may lead to increased vulnerability of the nervous system to subsequent injury and the development of chronic pain states for which there is some clinical evidence (Jensen et al, 1985).

Loss of inhibitory mechanisms may contribute to pathological processing after nerve injury. It has been suggested that degeneration of neurones is triggered by intense stimulation associated with ectopic discharges in damaged neurones (Sugimoto et al, 1989; Nachemson & Bennett 1993). If the affected cells are inhibitory inter-neurones there may follow significant alterations in the spinal processing of afferent information. NPY and galanin may be released throughout the dorsal horn and act in an inhibitory manner to compensate for the loss of inhibitory mechanisms due to transynaptic degenration. Additonally, it has been suggested that galanin may be tonically released primarily from glomeruli in lamina II after axotomy

presynaptically to inhibit C-fibre induced excitability (Randic et al, 1987; Zhang et al, 1995b) and that NPY may have a similar inhibitory action in the lower region of the dorsal horn (Kashiba et al, 1994; Zhang et al, 1995b). It has been suggested that NPY may act at autoreceptors to inhibit it's own release after transection of a peripheral nerve (Kashiba et al, 1994 ; Zhang et al, 1995b). Supporting this there is evidence that NPY released from the sympathetic nerve terminals to blood vessels acts as a negative feedback to inhibit it's own release and also that of noradrenaline (Stjarne & Lundberg 1986; Wahlestedt et al, 1986).

An alternate hypothesis is that NPY is responsible for some of the reported organisational changes in the dorsal horn following peripheral nerve injury. Neuropeptides have been shown to induce changes in neuronal cytoarchitecture as reviewed by Lipton & Kater (1989). Several studies have suggested that neuropeptides have neurotrophic actions influencing neuronal survival, phenotype and neurite sprouting (Lipton & Kater 1989; Pincus et al 1990; Mattson 1988). White and Mansfield (1996) found that the dendritic outgrowth property of dorsal root ganglion neurones cultured 10 days after axotomy of the sciatic nerve was increased by NPY. They suggested that NPY as well as other peptides such as VIP may contribute to the growth of primary afferents after nerve transection.

Although not directly relevant to the role of NPY centrally, it has been suggested that peripheral NPY contributed to hyperalgesia in the Seltzer & Shir model (Tracey et al, 1995a). They found that subcutaneous injection of Y_1 agonist into the hind paw exacerbated mechanical hyperalgesia but relieved thermal hyperalgesia. whereas subcutaneous injection of Y_2 agonist into the dorsum of the hindpaw significantly exacerbated mechanical and thermal hyperalgesia. They concluded that the effect of NPY on mechanical hyperalgesia was mediated by both Y_1 and Y_2 receptors while the effect of NPY on thermal hyperalgesia was mediated by the Y_2 receptor type. Surgical sympathectomy relieved the hyperalgesia and eliminated the effects of NPY and its agonist. This suggested that NPY contributes to

peripheral hyperalgesia by actions at receptors located on postganglionic sympathetic terminals.

6.4. CONCLUDING REMARKS

Before starting these experiments, it was unknown to me whether NPY would be easily detected in normal rats. It was surprising therefore to find an extensive basal presence of extracellular ir-NPY throught the entire dorsal horn and most of the ventral horn of normal rats. This basal presence was found in the absence of any active peripheral stimuli and was unaltered by spinalisation in these experiments. Because of the known coexistence of noradrenaline and NPY in some brainstem neurones, a greater influence from the descending fibres was expected. It is posssible that anaesthesia may have dampened the activity of neurones in the brainstem. Electrical stimulation of primary afferents of the sciatic nerve did not result in release. This was not unexpecterd since NPY is not present in primary afferents but it was still possible that an input in primary afferents could evoke intraspinal release of NPY by activating spinal or supraspinal proceses. These results suggested that this did not occur and that the extensive basal presence of ir-NPY originated from intrinsic neurones. This widespread tonic release suggests a role for NPY in the spinal cord. It would be of interest to know what this role is and whether it differs in neuropathic animals.

With neuropathic rats, an additional zone of release of ir-NPY was evident in the ipsilateral spinal cord but not in the cord contralateral to the nerve injury. This release was obtained in the absence of peripheral stimuli. Before starting these experiments, I thought it may be necessary to evoke releasé by nerve stimulation in these animals as peripheral stimuli are required to demonstrate the behavioural

abnormalities that develop such as allodynia and hyperalgesia. It is probable that this spontaneous release was due to ectopic impulses in primary afferents and thus the release of this neuropeptide might contribute to the abnormal pain state. The former could be further investigated by using local anaesthetic block both proximal to the ligature and proximal to the dorsal root ganglion to interrupt these impulses and hence determine which block abolishes the basal release in the spinal cord.

To investigate the probable role of NPY in the behavioural syndrome produced by nerve ligation several experiments could be performed. Firstly, it would be useful to study the release of NPY during the time course of this model to determine whether the release correlates with the behavioural changes. Additionally, it would be of interest to use selective antagonists of NPY before during and after the nerve ligation to investigate the role of NPY in the development of abnormal behaviours. It would be useful to study this neuropathic model in mice as mice which lack NPY have been developed to investigate the role of NPY in feeding behaviour (Erickson et al, 1996). In the absence of good antagonists, the role of NPY could be investigated by ligaturing the sciatic nerve of mice which lacked this gene to express NPY. Alternatively, if it is possible to disrupt the NPY gene in rats, it would be of interest to ligature the sciatic nerve to determine whether the absence of NPY alters the behavioural changes associated with the neuropathic model.

It would also be of interest to investigate the release of ir-NPY in the other neuropathic models and investigate the minimal insult that needs to occur to the nerve to result in NPY release in this new zone in the mid and deep dorsal horn. Additionally, the release of NPY in rats with complete nerve transection should be compared to the results obtained with partial nerve injury. Future experiments should also include the application of neurotophin-3 to the injury site, to determine whether chronic application would alter the release of ir-NPY in the complete transection and partial nerve injury models. In determining the role of NPY in neuropathic pain, it may be more appropriate to consider the co-action of the other neuropeptides with

which NPY is co-localised such as galanin. More studies investigating the receptor changes which occur after nerve injury may help to define the proposed inhibitory role of NPY. Finally, further investigation is required to determine whether NPY's role is to alter neuronal excitability or to promote structural changes during the regenerative phase.

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PUBLICATIONS

The following publications are derived from experimental work with which I was involved during my postgraduate study:

1. Duggan, A.W., Riley, R.C., Mark, M.A., MacMillan, S.J.A., Schaible, H.-G.
"Afferent volley patterns and the spinal release of immunoreactive substance P in the dorsal horn of the anesthetized spinal cat" *Neuroscience,* 65 No3 (1995) 849-858

2. Colvin, L.A., Mark, M.A., Duggan, A.W.
"Bilaterally enhanced dorsal horn postsynaptic currents in a rat model of peripheral mononeuropathy" *Neuroscience Letters*, 207 (1996) 29-32

3. Mark, M.A., Colvin, L.A., Duggan, A.W
"Antibody microprobe studies of release of immunoreactive neuropeptide Y in the spinal cord of the neuropathic rat" *J.Physiology Proceedings,* 495 (1996) 21P .

4. Colvin, L.A., Mark, M.A., Duggan, A.W.
"Antibody microprobe studies of ir-galanin release in the spinal cord of the neuropathic rat" *British Journal Anaesthesia,* 78 (1997) 462P

5. Mark, M.A., Jarrett, B., Colvin, L.A., MacMillan, S.J.A., Duggan, A.W.
"The release of immunoreactive-neuropeptide Y in the spinal cord of the anaesthetized rat and cat" *Brain Research (in press)*

COMMUNICATIONS

The following communications to learned societies were made:

Poster : **"Pain Mechanisms and Management"**
[Pain Research Institute, Liverpool-September 1995]
"Antibody microprobe studies of release of immunoreactive neuropeptide Y in the spinal cord of the rat"
Mark, M.A., MacMillan, S.J.A., Colvin, L.A., Duggan,A.W.

Poster : **"Pain Mechanisms and Management"**
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"Unexpected Electrophysiological Findings in a Rat Model of Peripheral Mononeuropathy"
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Oral : **Physiological Society meeting**
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"Antibody microprobe studies of release of immunoreactive neuropeptide Y in the spinal cord of the neuropathic rat"
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"Enhanced synaptic activity in the spinal cord in a rat model of peripheral mononeuropathy"

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